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**DINOFLAGELLATE TOXINS RESPONSIBLE FOR CIGUATERA FOOD POISONING**

**Annual Summary Report**

Donald M. Miller

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## SUMMARY

Ciguatera is a syndrome occurring in humans who have become intoxicated from eating poison fish. Fish spontaneously accumulate the toxin through the food chain or directly from eating toxic dinoflagellates. Previous research points to the presence of multiple toxin involvement. In addition to the establishment of facilities, this contract requires the growth of sufficient quantities of three different species of dinoflagellates to allow purification of milligram quantities of toxins for delivery to the U.S. Army Medical Research and Development Command. In this first year of the contract, necessary personnel were acquired and equipment setup to grow the dinoflagellate *Gambierdiscus toxicus* in mass culture. Purification of the products of these cultures is in progress

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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## BODY OF THE REPORT

### STATEMENT OF THE PROBLEM

Ciguatera is a form of food poisoning in humans resulting from the ingestion of toxic fish. It is suspected that the "ciguatera syndrome" is the result of several toxins accumulated by fish through the food chain from dinoflagellate progenitors.

Dinoflagellates produce a variety of toxins, some of which are ion channel inhibitors. In the case of ciguatera, the toxins can be accumulated through the food chain and stored by fish which are eventually consumed by humans. The ingestion of toxic fish produces a variety of digestive and neurological symptoms and sometimes death. The long term neurological effects are exacerbated by ethanol imbibition. At the present time, there is no adequate assay system for the detection and identification of the toxins. The structure, mode and site of action of these toxins is unknown. There is at present no known prophylactic or ameliorating treatment for ciguatera intoxication.

The acquisition of reasonable amounts of purified toxins would help to unravel the molecular structure of the toxins, their physiological actions and help to develop effective prophylactic treatment and effective countermeasures against the actions of the toxins.

We proposed to grow toxic dinoflagellates in mass culture for an extended period of time. After the cultures reached maximum growth they would be harvested and crude extracts made. The crude extracts would be assayed by mouse (LD<sub>50</sub>) and an isolated bioassay preparation. Crude extracts would be purified by HPLC. The purified toxins would be assayed the same as the crude and samples of purified toxin delivered to the US Army Medical Research Institute of Infectious Diseases.

Our previous work on the ciguatera problem has been funded by the U. S Food and Drug Administration and is currently supported by the College Sea Grant Program. One major consequence of this research was the establishment of the SIU Culture Collection of Tropical Dinoflagellates.



## BACKGROUND INFORMATION

**CIGUATERA POISONING** Ciguatera poisoning is a syndrome which occurs following the ingestion of certain tropical marine reef-fishes that sporadically acquire toxicity. It is one of nine known forms of ichthyosarcotoxism: poisoning of humans resulting from eating fishes which contain poison within their musculature, viscera or skin (1). Halstead (2) has implicated over 400 species of marine fishes as carriers, most, if not all, of which are an integral part of the food web of coral reefs associated with oceanic islands within a circum-global belt from 35°N to 34°S (2, 3).

TABLE 1.  
CIGUATERA SYMPTOMATOLOGY AS PERCENT OF CASES

Symptom	St. Croix n =5 Tackett et al. 1982	St. Thomas n =33 Tackett et al. 1982	Miami, Fla. n =129 Lawrence et al. 1980	South Pacific n =3009 Bagnis et al. 1979
Diarrhea	81	91	76	71
Abdominal Pain	54	39	--	46
Vomiting	40	70	68	38
Pain and Weak- ness in Lower Extremities	81	58	--	--
Dysesthesias				
Circumoral	38	36	54	89
Extremities	40	33	71	89
Temp. Rev	23	36	--	88
Dental	13	24	--	25
Itching	66	58	48	45
Arthralgias	30	52	--	86
Dizziness	--	21	47	42
Bad taste	43	27	--	--
Myalgias	30	30	86	82
Chills	30	24	--	59
Sweating	30	18	24	37
Headache	45	06	--	59
Rash	--	09	--	20
Paresis	--	00	--	10
Excessive Salivation	--	06	--	19

**THE CIGUATERA SYNDROME** The symptoms that occur after eating toxic fish typically includes both gastrointestinal and neurological manifestations. Typical symptomatology in humans has been

reviewed and summarized by several authors (4, 5, and 6: see Table 1.).

Earliest symptoms of intoxication usually include gastro-intestinal upset, which may last for several hours or weeks. Moderate to severe intoxications usually produce neurological symptoms which may last weeks to months. Thus, it is quite clear that intoxication affects the nervous system for extended periods of time. In some cases of severe intoxication symptoms have persisted for 25 years (7); death may result and usually occurs after several days. In a few cases, death has occurred within ten minutes and the fatality rate has been approximated as 12% (2) and 3% (5). It is suspected that a large number of ciguatera intoxications, some from eating frozen fish, are not recognized as such (8).

**MULTI-TOXIN INVOLVEMENT** A few early researchers suggested that the great variety of symptoms displayed by patients suffering from ciguatera and their inconsistent responses to certain clinical treatments indicated that there was more than one primary toxin causing the ciguatera syndrome (9,10), while Banner et al., (11) argued that ciguatoxin was the principal factor. Nevertheless, later studies coupled with the variability in results from testing of extracted fish tissues on a variety of preparations have emphasized the occurrence and importance of secondary toxins (12, 13, 14, 15, 16 and 17).

Hutner and McLaughlin (18) suggested that the zooxanthellae (dinoflagellates) which grow symbiotically in the bodies of some jelly fish, sea anemones, corals, and gorgonians might be the source of ciguatoxin. Banner, (11) argued that zooxanthellae were unlikely prospects because very few fishes eat coral and saxitoxin (STX = a paralytic shellfish poison which originates in certain dinoflagellates) differs from ciguatoxin both chemically and pharmacologically. However, Banner's conclusion regarding the dissimilarities of the toxins was not based on tests of zooxanthellae species but rather on the assumption that all dinoflagellate toxins would be expected to be similar.

Yasumoto et al., (14) provided evidence that ciguatoxin was of exogenous origin and was not a metabolic product of primary consumers. These authors reported that an analysis of gut contents of *Ctenochaetus striatus* (a detrital feeder, exclusively) revealed a

portion, designated as unidentified particles, containing a high concentration of "ciguatoxin".

**DINOFLAGELLATES PRODUCING TOXINS** Dinoflagellates are eukaryotes in the botanical division Pyrrophyta (19). The forms thus far described which are toxic are photosynthetic, and comprise less than 2% of the known species (20). Numerous dinoflagellates are known to produce toxins and there are innumerable examples of suspected toxin producers. Baden (21) has classified toxic dinoflagellates into two general types with subdivisions under each and some examples (Table 2).

TABLE 2.  
CATEGORIES OF TOXIC DINOFLAGELLATES

Bloom Organisms (= Red Tide)		
Water Soluble Toxins		
	<i>Gonyaulax catenella</i>	STX, Saxitoxin
	<i>Gonyaulax excavata</i>	GTX, Gonyautoxins
Lipid Soluble Toxins		
	<i>Ptychodiscus brevis</i>	Brevitoxins
Ciguatera Organisms		
Both Water & Lipid Toxins		
	<i>Gambierdiscus toxicus</i>	Ciguatoxin
		Maitotoxin & others

**DINOFLAGELLATE TOXINS AFFECTING ION CHANNELS** The use of ion channel toxins has been crucial to uncovering the mechanisms of how ion channels work. It is well known that saxitoxin and gonyautoxins are inhibitors of sodium channels. Recently, it was discovered that brevitoxins also affect the sodium ion channels (22). We are now finding that there are toxins in the ciguatera group that affect calcium and sodium channels.

**DINOFLAGELLATE INVOLVEMENT IN CIGUATERA** That dinoflagellates are the source of ciguatera-toxins has been well documented. In the Pacific, Yasumoto and others (23, 24) obtained significant quantities of ciguatoxin from samples of detritus collected from dead coral near the Gambier Islands. The most toxic fraction of the detritus contained large numbers of a dinoflagellate, which he tentatively identified as "*Diplopsalis* sp.". Subsequently, Adachi and Fukuyo (25) named the organism *Gambierdiscus toxicus*. Yasumoto et al., (16) connected the production of toxin with both the dinoflagellate and toxic effects in mice (26). The dinoflagellate, *G. toxicus* has subsequently been isolated from ciguatera prevalent

areas near Japan (26, 27), and Hawaii (28, 29). McFarren and others (30) have provided accounts of ciguatera-like poisoning (*G. breve* ?) from shellfish collected from the west coast of Florida. Other investigators have published on *Gambierdiscus toxicus* from Florida (31). Tindan and his group (32) have grown in mass culture and extracted toxins from three dinoflagellate species which were isolated from areas of the Caribbean in which ciguatera intoxication was prevalent. Thus far, three particular dinoflagellates are implicated in the production of ciguatera toxins: *G. toxicus* in which four toxic fractions have been identified, GT-1, GT-2, GT-3, GT-4; *P. concavum* in which five toxic fractions have been described, PC-1, PC-2, PC-3, PC-4, PC-5; and *P. rathymum*(= *P. mexicanum*) in which one toxic fraction, PR-1, has been described.

#### EVIDENCE FOR STRAINS OF TOXIN-PRODUCING DINOFLAGELLATES

There is ample evidence to indicate that different strains of the same species of dinoflagellates produce different numbers and amounts of toxin (33, 34, and 35). There are also reports of loss of toxicity of dinoflagellates after culture. Under our conditions we have found that the initiation of mass cultures from unialgal or pure cultures has confirmed the strain differences but our cultures (*G. toxicus*) have produced toxins through continual subculturing for a period of 7 years and through over 30 large scale cultures. The same holds true for the other species.

**ISOLATION OF DINOFLAGELLATES TOXINS** Yasumoto (16) extracted toxic components from *G. toxicus* cells utilizing a boiling methanol extraction prior to doing a water-ether partitioning. The ether extracted portion was further treated with acetone to derive a toxic fraction. Since we have found this fraction to precipitate in very cold acetone we term it the ether soluble acetone precipitate (ESAP) fraction. Most cell isolation procedures used an initial partitioning of the cells with a water-ether mixture. The treatment of the water phase of the cell extracts have been similar by all investigators. After an acetone partitioning the filtrate is chromatographed to yield a water soluble toxic component (Figure 1).

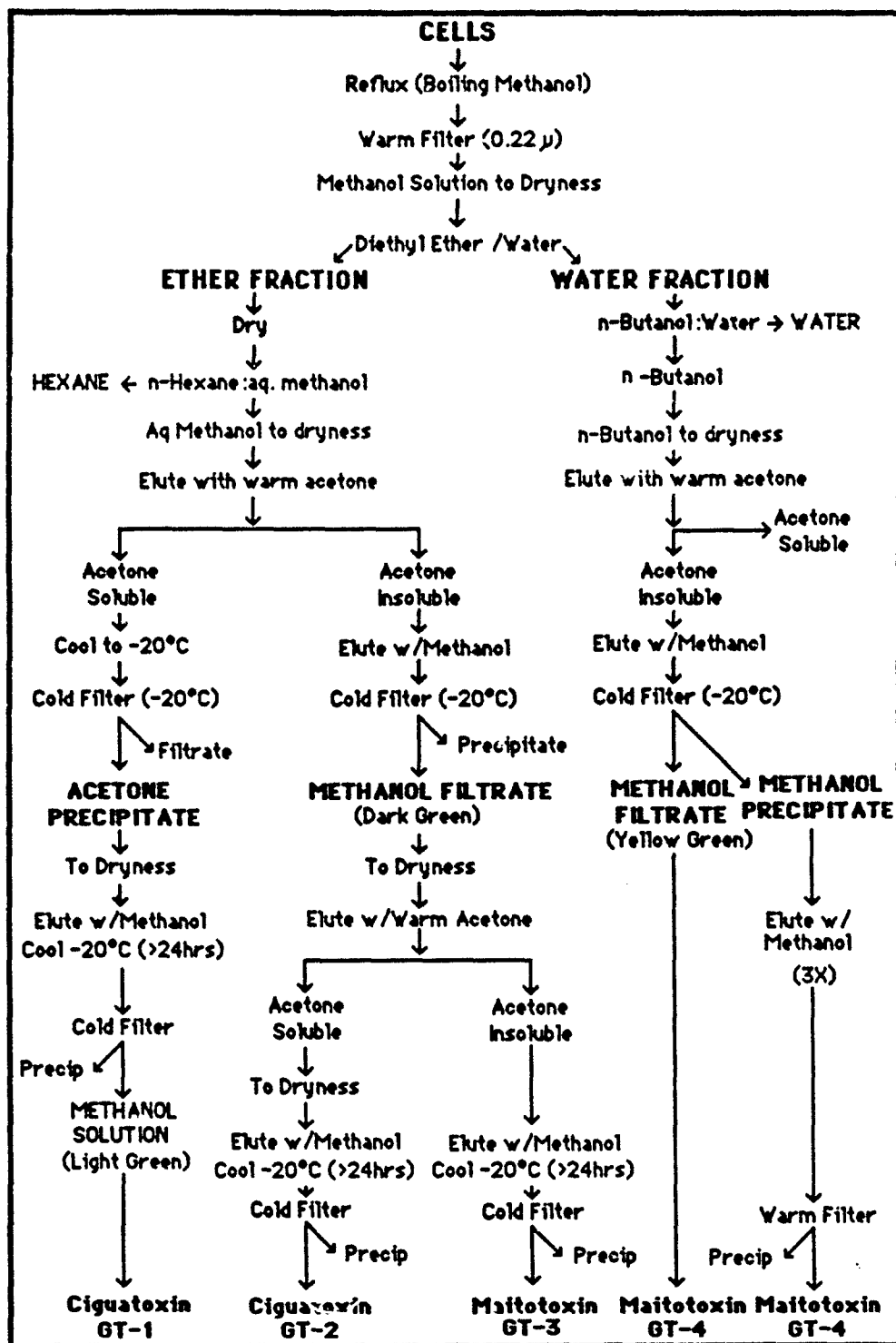


Figure 1. Schematic flow diagram of separation procedures for *Gambierdiscus toxicus*.

The treatments for the ether phase of the cell extracts have differed. Yasumoto used a technique which involved an acetone extraction of the ether-water phase. Bagnis and others (36) modified the technique to include a cold acetone treatment that resulted in both ether-soluble acetone precipitates (ESAP) and ether-soluble acetone filtrates (ESAF). Even though he utilized this separation procedure, he then combined the ESAP with the WSAP fraction. Tindall and his group discovered that when the ESAF material was kept in the cold acetone all of the toxic activity eventually precipitated out of the filtrate. Thus, they adopted acetone precipitation as a step in the procedure hoping to further purify the toxic component. The toxic fractions obtained by these procedures differ with the particular dinoflagellate species.

**FISH TOXINS** The studies of toxins from fish extracts are difficult to draw conclusions from for several reasons, the most critical of which is that, if there are multiple toxins in fish, determined by their diet, and any one toxin may have different effects on a variety of assay systems, then it is possible that the extraction of toxin from the same species of fish by investigators from different locales will produce different symptomatology, etc.

From the foregoing we conclude, that if we want to determine if a particular fish is toxic, we must have a test(s) or assay system(s) which is specific at a known level for each of the particular toxins which may be involved in the ciguatera syndrome. For this express reason we decided at the outset of our experimentation that the utilization of cell cultures would be the most productive approach.

Indeed, the same philosophy argued above for the diagnosis of toxic fish would apply to the treatment of the disease ciguatera. A different treatment would be called for were a person intoxicated with a sodium channel inhibitor rather than a calcium channel activator. Eventually, it would be ideal to have an assay system which would, in fact, reflect the number, kinds and amounts of toxins. The construction of these specific chemical tests is only possible, however, if one has reasonably pure toxin, which is separated from other toxins and identified.

These constraints have dictated our approach to the entire problem since we started our research in 1978, as follows:

1. Identify sources of the toxins.
2. Produce large amounts of toxic organisms.
3. Improve extraction techniques.
4. Find a sensitive bioassay(s) for screening.
5. Use bioassay to assist in purification.
6. Improve purification techniques.
7. Use purified toxins to:
  - a. investigate physiology.
  - b. investigate structure.
  - c. elaborate chemical assay system.

Our preliminary works (32, 37, 38 and 39) have established that at a minimum at least six different toxins and possibly a seventh are involved in the ciguatera syndrome:

1. Ciguatoxin
2. Maitotoxin
3. Slow acting toxin (unidentified)
4. Scaritoxin-like toxin
5. Okadiac acid
6. Fast-acting toxin I (unidentified)
7. Fast-acting toxin II (unidentified)

Clearly, the understanding of the entire problem of the toxins produced by dinoflagellates requires a definitive test or set of tests which will allow us to discriminate between the toxins and be able to quantify and follow them. Other desirable features of a test procedure would be 1) provide the same baseline for each of the toxins, 2) consume only a small amount of toxin, 3) require a small amount of time for the assay, and 4) be able to detect modifiers of sodium channels in membrane as well as calcium channels.

**SIGNIFICANCE** Firstly, it is apparent from Table 1 that the toxins are involved in a variety of short term symptoms for which people commonly present themselves to a doctor for treatment (e.g. diarrhea, headache, etc). In addition intoxication can result in prolonged disability or even death.

Another aspect of the toxins and one which has yet to be addressed is the long term effects on animals that is: are they

cumulative, are there storage sites, what are the affective sites, and how long before excretion? The reoccurrence of neurological symptoms years after intoxication would seem to indicate a retention of the toxin and/or toxic effects in the nervous system.

The toxins also include a novel calcium channel inhibitor (40, 41 and 42). In addition, there is the prospect of discovering other new and important ion channel inhibitors. Ion channel inhibitors have been essential to our present understanding of ion channel physiology and structure. Clearly, the identification, isolation and purification of individual toxins involving ion channels will expedite (1) an understanding of their structure, (2) allow the investigation of their physiological actions, (3) expedite the formulation of ameliorative and prophylactic treatments, and (4) allow the elaboration of a specific chemical assay.

#### APPROACH TO THE FIRST YEAR OF THE STUDY

**ACQUISITION OF EQUIPMENT AND SETUP** In the original proposal we had allocated three months for the acquisition of all of the equipment.

**GROWTH OF CELLS** The dinoflagellate cultures which we used for this project are part of the Southern Illinois University Culture Collection, housed in the Department of Botany. At present this collection houses strains representing dinoflagellate species isolated from "ciguatera community" areas of the British and U. S. Virgin Islands and other recently acquired species (see Table 5, in results section). Our stock cultures are routinely grown in 50 ml volumes in 125 ml Erlenmeyer flasks. The medium is ES Medium (43) made with natural seawater, with 1.5% soil extract added. These cultures are kept in refrigerator-type culture chambers at 27°C and 500 ft-c. cool white fluorescence illumination (either continuous or on a 16:8 light-dark cycle, depending upon the requirements of the particular species). Stock cultures are transferred every 7-10 days. Two generations of cultures are retained as back-ups to the new transfers. The subculturing and maintenance of triplicate cultures is labor intensive and requires approximately 20 manhours per week by an experienced person. In addition, all cultures are examined periodically by one of us to check for contamination. Currently we maintain stock cultures of over 153 strains of dinoflagellates. Preparation of the growth medium requires millipore filtration (0.45



or 0.22  $\mu\text{m}$ ) and sterilization of the sea water, sterilization of the flasks, compounding of the growth medium, inoculation and siting in the growth chambers. Conservatively, this requires approximately 20 manhours per week for a total of 18 carboys. Because the growth cycle for both the subcultures and the mass cultures takes four weeks, we initiate a mass culture every two weeks. It is critical for the cultures, to achieve the maximum toxin production, that they be harvested very close to the 30 day period.

With the two week cycling (starting in December 1987) we harvest and process 36 liters of toxic extracts every two weeks. Thus, during the entire growth period for any one species there are 72 carboys growing. The development of mass cultures involves transferring cells from stock cultures to a series of two liter fernbach flasks containing enriched seawater medium. After the early stationary phase of growth has been reached (approximately 15-20 days) each of these cultures are used to inoculate 18 liters of the same medium in 20 liter carboys. Mass cultures are grown under the same light and temperature regime as noted above and are aerated continuously in order to prevent  $\text{CO}_2$  depletion and to provide moderate agitation. Cells from small cultures are harvested by centrifugation or filtration. Cells from mass cultures are harvested by means of a Pelicon concentrator using 0.45  $\mu\text{m}$  membranes after cultures reach the early stationary phase of growth (30-35 days). If the culture has excessive amounts of slime it is first sieved before the use of the Pelicon.

**EXTRACTION OF TOXINS** The initial methods for the extraction of the toxins is very similar to what is currently being utilized by other investigators (especially those attempting to isolate toxin from fish tissues) so that, to some extent, we can compare the toxic fractions which we obtain with what is in the literature.

The cells are no longer extracted by refluxing in boiling aqueous methanol. Instead, extraction is now accomplished by crushing of cell, followed by sonication in methanol at room temperature. This elimination of the boiling methanol step was dictated by our finding in the results section. The methanol extracts are concentrated and subjected to liquid-liquid partitioning followed by cold acetone precipitation of the toxic components. This process is diagrammed in Figure 1.

**PURIFICATION** Further purification of the toxin will be either by thin layer chromatography (TLC), silicic acid chromatography or high pressure liquid chromatography (HPLC). Eluting solvents for column chromatography consist of chloroform-methanol (1:1), and chloroform-, methanol-water mixtures (9:1:1). The eluting solvent for HPLC is methanol (100%).

**HPLC PURIFICATION** We have been working out the methodology for the separation of toxins with a minimum of loss on HPLC. We feel that a preparative HPLC separation approach conserves toxin, saves time, and is cheaper in the long run as opposed to regular column chromatography. We presently perform the initial purification on C-18 columns with methanol as the solvent. Separation is achieved using three HPLC associated with a single computer controller system. All three are Waters Company instrumentation and consists of a Delta 3000 Preparative-Semipreparative HPLC and a Model 300 Analytical system all interlinked with SIM modules to an 820 Controller System. While we use C-18 columns in each system, the sizes differ, having 15  $\mu$  in the Preparative and Semipreparative and 10  $\mu$  or 5  $\mu$  in the Analytical.

**MOUSE BIOASSAY** The mouse bioassay is the officially recognized toxicity assay for ciguatera recommended by the Official Organization of Analytical Chemists and the FDA. In addition, it provides a base line against which we can compare our isolated preparation assays.

The carrier for toxic extracts is normal saline containing 0.5 ml of a 1% Tween-60 solution. Toxicity is determined by an intraperitoneal injection of 0.5 ml of a suspension of extract into approximately 20 g mice (Strain CRE:CD:BR:ICR). Toxicity is defined as death of the mouse within 48 hours. LD<sub>50</sub> values are calculated according to the method of Weil (44). Four dosage levels are used with three repetitions at each level. The LD<sub>50</sub> is calculated from moving average interpolation tables.

**THE ILEUM ASSAY PROCEDURES** We have utilized the terminal portion of the guinea pig ileum to assay dinoflagellate toxins. The overall setup for the ileum assay is shown in Figure 2.

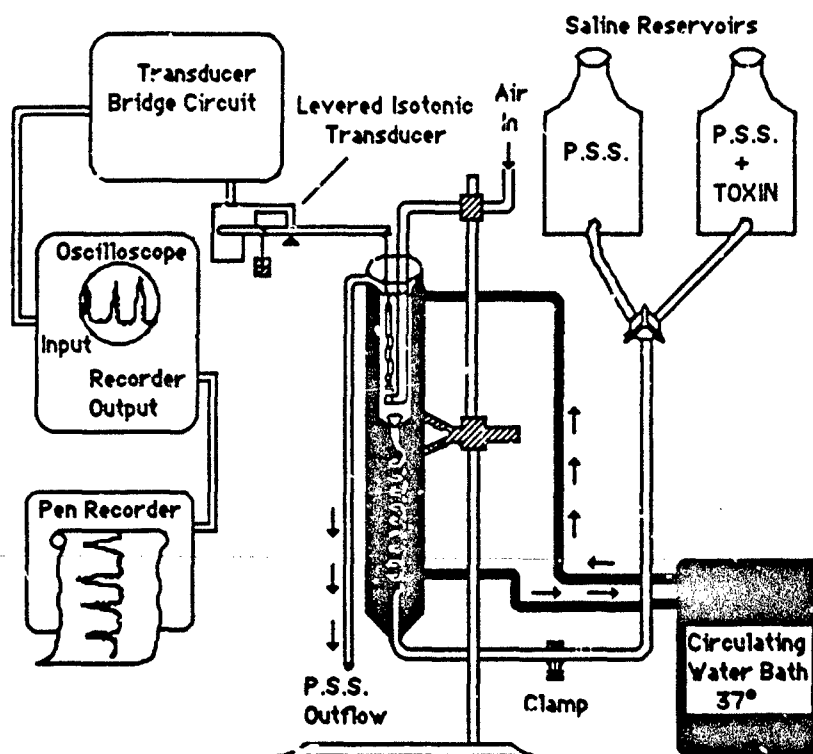


Figure 2. Schematic diagram of the equipment setup for the guinea pig ileum preparation. Individual components are discussed in the textual material.

**Guinea Pig Ileum.** Female guinea pigs (350-600 g) were sacrificed by a cervical dislocation. A 2-4 cm segment of the terminal ileum was removed and placed in physiological saline solution (PSS) at 37°C. If the tissue is not going to be used for a while it may be stored in the refrigerator in saline solution. When the ileum is excised from the animal, great care must be utilized to keep stretching and mechanical trauma at a minimum. Thus, when you cut, make one smooth cut with a scalpel. The terminal part of the ileum in most mammals contains a smooth muscle sphincter. In the normal state the sphincter muscles are quiescent and positioned such that the sphincter is closed. This is in contrast to the upper part of

the ileum which is normally actively contracting, moving chyme down the tract. The closed condition of the ileocaecal sphincter prevents the back up of food material from the caecum from entering the ileum. The terminal portion will, however, respond to exogenously applied agonists. Hence its suitability for use in an assay system. If pieces of ileum too far away from the caecum are used in the assay, spontaneous activity is usually encountered. In this case it is difficult to tell if you stimulated the ileum or it was a spontaneous contraction.

**Physiological Saline Solution.** The physiological saline solution for the guinea pig ileum consists of the following (mM):

TABLE 3  
PHYSIOLOGICAL SALINE COMPONENTS

CHEMICAL.	mM	g/L	Stock g/L	amt/L
NaCl	136.9	7.014		weigh out
KCl	2.68	0.372	186.37	2.0 ml
CaCl <sub>2</sub>	11.84	0.277	55.5	5.0 ml
MgCl <sub>2</sub>	1.03	0.095	23.8	4.0 ml
NaHCO <sub>3</sub>	11.9	0.084	8.4	10.0 ml
KH <sub>2</sub> PO <sub>4</sub>	0.45	0.178	89.1	2.0 ml
glucose	5.55	1.980		weigh out

Special care must be used in the preparation of the saline solution. Altered sodium-potassium ratios may result in spontaneous contractions in the ileum preparation. Elevated calcium may result in increased contractility and exaggerated inotropic responses.

**Tissue Bath.** After removal from cold storage, the excised terminal ileum is allowed to come to temperature in saline for 15 min then a one cm segment is cut and inserted in an Anderson type tissue chamber (45) modified as suggested by Bartelstone (Figure 2). This bath consists of two sections, one for the tissue itself and a second one underneath with a spiral conenser coil for warming the saline before it reaches the bath. Perfusate is introduced into the bottom of the spiral coil and circulates up thru the coils and into the tissue chamber. The volume of the spiral and vertical inflow chamber has a minimum of four times the capacity of the tissue chamber. Thus allowing adequate time when flushing the bath for temperature control of the perfusate. A button valve at the bottom of the tissue bath blocks circulation of physiological saline once the flow from the reservoir is cut off. Perfusate leaves the tissue bath

by way of an overflow outlet at the top. The tissue is held by means of a glass tube that is mounted on a metal bracket at the top of the chamber, and extends down into the chamber to just above the button valve at the bottom of the tissue chamber. A small hole in the tube, at the point where the tissue is secured, allows aeration of tissue and perfusate. Alternately air can be bubbled in through the secondary opening at the base of the chamber just above the button valve. The tissue is then extended upwards towards the top of the tissue chamber where it is connected by means of a suture, to a recording device for registering the contractions of the tissue.

**Water Circulating Bath.** The guinea pig ileum is extremely temperature sensitive and changes as small as  $0.3^{\circ}\text{C}$  may affect its contractility and therefore your results. For this reason you must have a circulating water bath or some other means of controlling the temperature. The circulating water bath that we presently use is Fisher Model M8000, Isotemp, Constant Temperature Circulator.

**Tension Transducer.** The ileum is connected to a locally-made device which is a true, isotonic-tension transducer. The counter weight we use is 2 grams. Movement of the balance beam interrupts a light supply from a T-24 car bulb to a photoresistor. The resulting change in current in a bridge circuit is proportional to the shortening of the ileal longitudinal muscle. Practically, the use of an isometric transducer or any kind other than a levered system is not recommended. The ileal tissue is very fragile and when it develops too much tension it literally tears itself off of the hook. Even small tears in the tissue change your baseline and ruin an assay.

**Amplification.** Amplification of the transducer signal is achieved by the use of a DC oscilloscope with a pen output. In our case we are presently using a Nicolet NC-110 digital oscilloscope. The use of the oscilloscope allows us to compensate for different length of tissue and thereby utilize the full range of the chart recorder for our records. We selected the Nicolet to replace a Tektronix 502A when the latter was broken. Our choice was dictated by the requirement for the amplification system to accept a differential input and have a DC level pen recorder output.

**Recorder System.** For storage of records and recording of data for calculations we chose an inexpensive chart recorder (Fisher Recordall Model 5000). Any good DC level recorder can be used, but a ten inch recorder makes the task of measurement much easier.

**Agonists.** The ileum has been shown to respond to acetylcholine, histamine, substance P, epinephrine, and several other compounds. Indeed, it has varying sensitivity to each of these compounds. We have found that the toxic fractions will give different inhibitions with different agonists.

**Protocol for Reversible Toxins.** The first protocol followed is utilized when there is only a reversible toxin in the extract. Initially a control series of acetylcholine or histamine stimulations is performed at different dosages to determine three doses which would give us values between 20 and 80% of maximum contraction of the ileum segment (Figure 3). That particular set of test doses is then utilized throughout the rest of the experiment. All subsequent responses of the ileum to agonist challenge are expressed as a percent of control values.

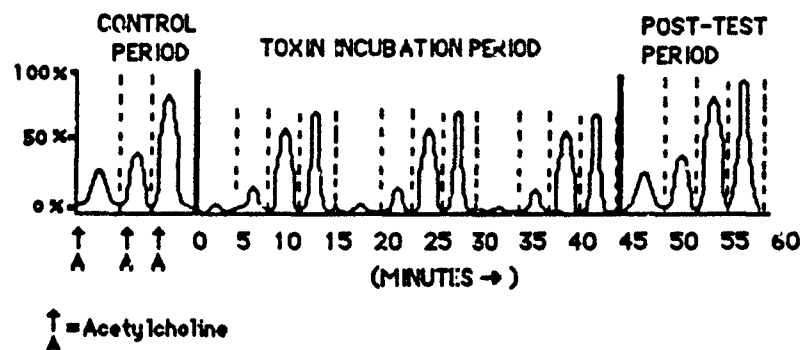


Figure 3. Schematic diagram of the methodology for the assay of a reversible toxin.

The toxin containing physiological saline solution is utilized for subsequent washes. Thus when testing for reversible inhibition, the toxin is always present in the PSS at the same concentration.

**Non-reversible Assay.** The second protocol is utilized when it is suspected that there only a non-reversible toxin in the extract. First a control series of histamine stimulations is performed at different dosages to determine a dose which would give us approximately 80% of maximum contraction of the ileum segment (Figure 4). That particular test dose is then utilized throughout the rest of the experiment. All subsequent responses to histamine are expressed as a percent of that control value.

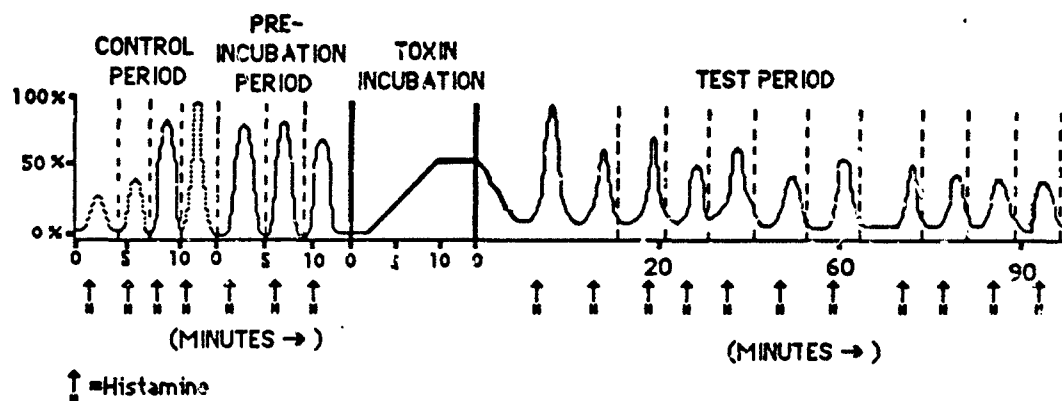


Figure 4. Schematic for the analysis of an irreversible toxin using the guinea pig ileum.

Once the control series is completed, the preparation may be subjected to a pre-treatment period during which the carrier for the toxin (e.g. methanol) is introduced as a second control. All experimental chemicals which are used with the toxin would be included in the pre-treatment period. After this pre-treatment period the toxin containing physiological saline solution and/or carrier is introduced into the bath. The preparation is bathed in toxin saline for a set time period (usually 15 min). When 15 minutes has expired the preparation may be challenged with histamine. After each challenge the preparation is washed in normal PSS. The preparation is then monitored for 90 min during which time test challenges of histamine are performed intermittently.

**Initial Protocol for Crude Abstracts.** The third protocol is utilized when it is suspected that there are both a reversible and a non-reversible toxin with a long delay period (maitotoxin) in the extract. First a control series of histamine stimulations is performed at different dosages to determine a dose which would give us approximately 80% of maximum contraction of the ileum segment (Figure 5). That particular test dose is then utilized throughout the rest of the experiment. All subsequent responses to histamine are expressed as a percent of that control value. the toxin containing physiological saline solution is introduced into the bath. The preparation is bathed in toxin saline for a set time period (usually 15 min) during this period the preparation is challenged once or twice with histamine. After each challenge the preparation is washed in toxin containing PSS. Once 15 minutes has elapsed, normal saline washes are resumed. The preparation is then monitored for

90 min during which time test challenges of histamine are performed intermittently.

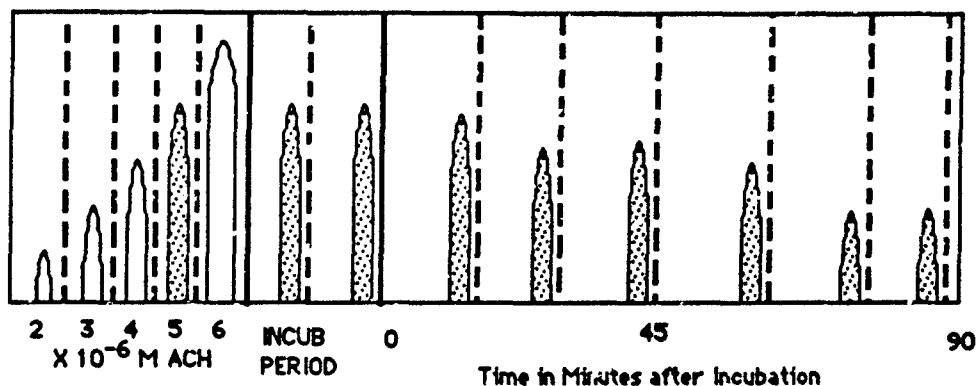


Figure 5. Schematic diagram illustrating methodology for using the ileum assay when both an irreversible toxin and a reversible toxin is present.

**TESTS FOR PURITY OF TOXINS** The easiest manner to test for purity of the toxin is to run in a recycle mode on the analytical HPLC to determine if after a given number of recycles the single peak remains or resolves into more than one peak. We will use several criteria for testing for purity: first the presence of single peak after recycling on analytical HPLC; second, migration on TLC plates in different solvent systems, and structural data from Nuclear Magnetic Resonance.

**NMR SPECTROSCOPY** At the present time Nuclear Magnetic Resonance (NMR) would provide us with the most informative data and yet is non-destructive to the sample analyzed. For these reasons it is the method of choice. Samples of purified toxins will be sealed in special, thin-walled, small-bore, NMR sample tubes from Wilmad Glass Co.

Two state of the art NMR instruments are presently available for our use: specifically a 300 MHz Varian VXR-300 and a 500 MHz Varian VXR-500 multinuclear spectrometer system. Both instruments operate in the pulse Fourier Transform mode and are equipped with a liquid helium VXR superconducting magnet and acquisition hardware. Both have H<sub>2</sub> fixed frequency lock system with 5 mm broad band computer switchable probe. Multiple probes



to two chambers accepting, 5 mm (narrow bore) and 10 mm (medium-wide bore) tubes for  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$ ,  $^{15}\text{N}$ ,  $^{19}\text{F}$  and other nuclei. Quadrature detection. Homo- and hetero-nuclear decoupling with spectral limits of 100 to 100,000 Hz. Variable temperature control ( $-70^\circ$  to  $140^\circ$ ) under computer control for all probes. VXR data station with dual high density disks. The VXR-300 operates at 300 MHz  $^1\text{H}$  resonance and is presently used primarily for  $^{13}\text{C}$  analysis. Automatic performance of standard relaxation experiments, as well as data recording is accomplished by an associated computer. The chemical shifts recorded are then interpreted for structure.

**PACKAGING OF TOXINS FOR SHIPMENT** Purified toxins in 100% methanol will be placed into vials, concentrated to almost dryness under nitrogen gas, sealed and labeled for shipment.

**ACQUISITION OF TOXIC DINOFLAGELLATES** Since the inception of our work on Ciguatera in 1978 we have conducted an extensive survey of ciguatera endemic regions of the British and United States Virgin Islands which resulted in the collection of 46 species of dinoflagellates. Sixty-five strains representing 18 of the most conspicuous epiphytic, benthic and planktonic species were isolated and brought into unialgal culture, harvested, and subjected to our standard extraction procedures. We proposed to continue our yearly survey for toxic dinoflagellates.

**OVERALL OBJECTIVES FOR THE FIRST YEAR** Our first years objectives can be summarized as follows:

- (1) Mass culture of *Gambierdiscus toxicus*.
- (2) Extraction of crude toxins from mass cultures.
- (3) Bioassays each of the isolated toxins.
- (4) Purification of toxic fractions using preparative HPLC.
- (5) Investigation of factors affecting the stability of stored toxins.
- (6) Quantization of toxins by weight and bioassay.
- (7) Examination of toxins by NMR.
- (8) Continuing investigation of effects of ethanol on toxicity.
- (9) Delivery of toxic fractions to U. S. Army Medical Research Institute of Infectious Diseases.
- (10) Continue to search for additional toxic dinoflagellates for addition to the SIU culture collection.
- (11) Maintain the SIU culture collection.
- (12) Investigate factors which improve toxicity.

## RESULTS

•**ACQUISITION AND SETUP OF EQUIPMENT** While the official starting date of the contract was listed as 1 December 1986, clearance to officially spend funds was not received through administrative channels until 5 Jan 1987.

Approval of positions - The hiring of any new personnel required the submission of requests through the affirmative action channels within the university. These applications were made and approval for the positions were received on 18 February 1987.

Approval of equipment - In the contract proposal we had not anticipated that there would be a 30 to 90 day delay in the "approval to purchase equipment" process. In order to save time we requested (2 Dec 86) and received permission from the contract specialist to initiate the purchasing process with the provision that final purchase would not be made until approval from the contractor was obtained. The last item to be approved was the growth chambers. Final approval for these items was not received until June, 1987. It was also stipulated that dates for the deliverables would have to be adjusted for the time delay in the acquisition of equipment which would be introduced by the contractor.

The large chambers which were due to be operational were held up an additional time period due to construction problems (installation of a cooling tower and recirculating system). Two of the chambers became operational on 15 November 1987.

•**LARGE-SCALE CULTURING OF *GAMBIERDISCUS TOXICUS*** Our harvesting activity is best summarized in Table 4 on the next page.

•**PURIFICATION OF TOXINS** Recently we have conducted experiments to examine the affects of solvents and storage conditions on semipure maitotoxin. These studies revealed that whenever there is more than 10% water in the solvent, the toxicity, as determined by bioassay, degrades rapidly with time (approximately two weeks). From these kinds of experiments, we have been able to specify storage conditions, such that we can retain toxicity for more than three years (the time length of the study).

Because of the reports that ethanol imbibition exacerbated the effects of ciguatera intoxication, even for years after the intoxication, we began investigating the effects of ethanol on the apparent toxicity. We have found that on our in vitro bioassays, ethanol does indeed enhance the apparent toxicity of maitotoxin (GT-4) when compared with methanol and other solvents. We intend to continue our studies along these lines with the other toxins.

TABLE 4  
CULTURES HARVESTED

CULTURE DESIG.*	DTE STARTED	VOL. (ltrs)	YIELD		
			gm	Dry Wt	Tot Dry Wt
GT350-4A87D	27Mar	227.0	101.6	12.2	12.2
GT350-6A87D	13May	243.0	99.9	13.0	25.2
GT350-7A87D	13May	159.0	77.6	9.20	34.4
GT350-7B87D	15Jun	70.0	17.50	2.80	37.2
GT350-8A87D	8Jul	65.0	18.6	2.60	39.8
GT350-8B87D	8Jul	284.0	130.0	17.9	57.7
GT350-9A87D	22Aug	42.0	19.2*	2.7*	**
GT350-9A-F	22Aug	156		18.27	75.97

Explanation of numbering system- Because we will be handling a large number of mass cultures we have revised our designating system. The first part of the designation is the initials and strain number of the culture then a dash and a number which represents the month in which the culture was harvested, following this a letter represents the sequence of harvest within that month, followed by a two number designation for the year. Finally, there will be a letter code at the end to designate the stage of processing of the sample. \* =estimated \*\*=used to directly inoculate carboys for GT350-9A-F, experimental run to examine faster culture sequence.

In order to examine those factors which affect the loss of toxin in storage and to determine which solvent would be best for separation of the toxin, we previously setup experiments with the water soluble extract. The procedure was to aliquot the same amount of water soluble extract into different vials, blow the toxin dry with dry nitrogen gas, and then take it up into the solvent being tested. The test-solvent was pipetted out into a separate vial to be blown dry again. Both vials then received the original amount of methanol solvent. A control vial served to establish a killing dose for the ileum. At time intervals after the initial aliquoting, ileum assays were conducted on the samples. The solvents examined were

methanol, ethanol, acetonitrile, methanol plus water and acetone. In addition to solvents, two other factors were examined in the experiments. These were acid versus basic conditions and light versus dark. All samples except the light vial (stored at room temperature) were stored in the freezer and aliquoted at intervals for testing on the ileum preparation.

The "factors" studies were designed to give us some appreciation for those extraction conditions which would minimize the loss of toxin and allow us to select the best solvent for purification. Similar to previous work on a much smaller scale, the results indicated that the length of time the toxin was in water (out of a lipid medium) and in the light were the largest factors in the loss of the toxin. One surprising result was that there was a component of toxin extracted from the water soluble extract by acetonitrile that was not detected when semi-purified toxin was extracted (or it was so small that it was not detected in our previous experiments which were done on an analytical instrument).

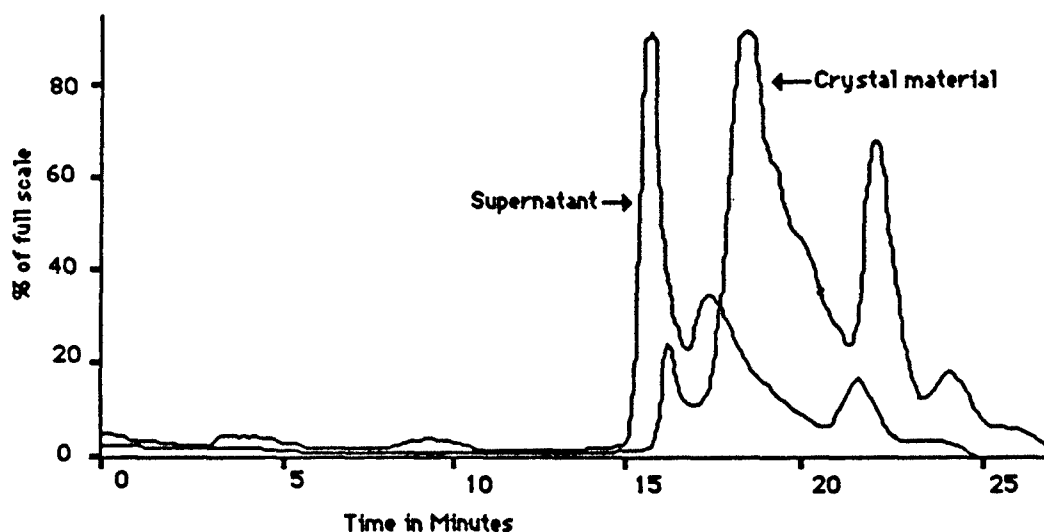


Figure 6. Semi-preparative HPLC chromatogram of crystalline supernatant (X) and re-suspended crystalline material isolated from water soluble extract (Y). Sample application was 25  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

**MOUSE BIOASSAYS** In addition to the routine assay of all mass cultures, we have built into our program an examination of ten different clones of *G. toxicus*. The growth characteristics have been described in a later section in this report, but the LD<sub>50</sub> data is tabulated in Table 5 following.

**TABLE 5**  
**A COMPARISON OF TOXICITY (LD<sub>50</sub>) OF CRUDE METHANOL EXTRACTS**  
**OF GEOGRAPHICALLY SEPARATED CLONES OF *GAMBIERDISCUS TOXICUS***

STRAIN (CLONE)	CELLS DW mg	METHANOL EXTRACT mg	LD <sub>50</sub> mg per Kg-Mouse	MOUSE UNITS	TOTAL M.U. mg	M.U. per mg/cells
GT-175	641	364	1.00	0.020	18200	28.39
GT-350 4A	5000	1908	5.75	0.115	16591	3.32
GT-350 6A	5000	1896	5.75	0.115	15878	3.18
GT-350 7A	5000	1800	6.25	0.125	14400	2.88
GT-350 7B	2800	900	5.00	0.100	9000	3.21
GT-350 Cb	25460	8280	5.00	0.100	82800	3.25
GT-135	127	63	14.73	0.295	213	1.68
GT-851	1542	563	54.75	1.095	517	0.34

**•PURIFICATION OF LARGE SCALE CULTURE PRODUCTS**  
Examination of our studies of extraction procedures pointed to a considerable loss of toxin. We concluded that any shortening of the procedures and elimination of water would considerably cut down on toxin loss. Therefore we initiated studies which would allow us to proceed to HPLC as expeditiously as possible. Accordingly we initiated four studies directed at (1) examining the separation of toxins on the semi-preparative system preliminary to application of larger amounts the the preparative system, (2) concomitant separation of peaks on the analytical system, (3) application of trial amounts to the preparative system, and (4) experimentation to eliminate water and shorten the separation process. As stated in the previous quarterly, preliminary runs indicated that the peaks of the crude toxin distribute differently on the semi-preparative column than they do on the analytical.

This is probably due to the fact that the resin size is different on the semi-preparative unit as opposed to the analytical system (15 versus 10 microns). Attempting to run the toxin on the semi-preparative HPLC with a mixture of methanol and acetonitrile resulted in blocking of the column. Further investigation of this phenomenon indicated that crystallization was occurring within the column.

Experimentation with the ratio of water to methanol in the medium demonstrated that crystallization could be induced in the water extract simply by altering the concentration of methanol. Once we learned the mechanism of formation, crystals were easily induced. Figure 6 is a diagram of semi-preparative HPLC recordings of both the crystal supernatant and crystals put into solution. Crystals were separated from the extract and tested on mice and the ileum, with negative results. We concluded that the crystals are products of non-toxic components. Nevertheless, this procedure may still be extremely beneficial as a rapid clean-up method for the crude fraction.

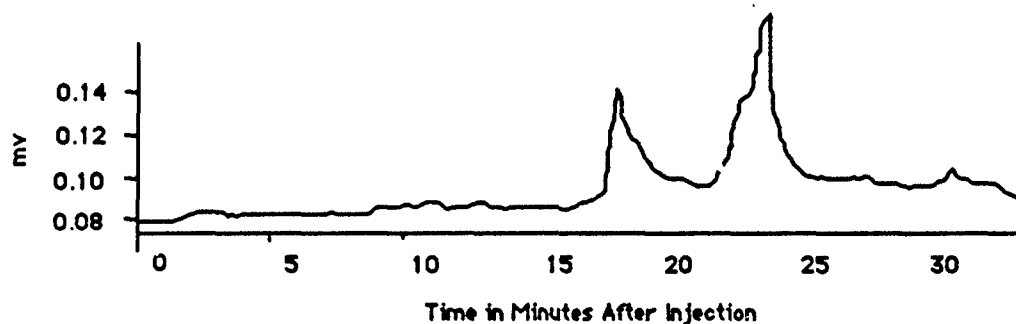


Figure 7. Semi-preparative HPLC chromatogram of water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

The original water soluble extract when chromatographed on the semi-preparative HPLC indicates a composite of many peaks in two distinct groups (Figure 7 above). Five hundred  $\mu$ l of this water soluble extract was dried down and submitted to acetonitrile extraction.

The result was an ACN-insoluble and an ACN-soluble division of the original water soluble extract each of which was dried down and diluted up to 500  $\mu$ l with dry methanol. When tested on the ileum (10  $\mu$ l sample directly in the bath of 10 ml of saline) both fractions are toxic, but produce subtle differences (Table 6, lines 2 & 3, page 36). Subsequent second and third extractions of the original water soluble extract with ACN are not toxic to the ileum (Table 6, lines 4 & 5). These results led us to conclude that there are in fact two or more toxins in the water soluble extract and the ACN-soluble fraction is perhaps less polar than the ACN-insoluble portion.

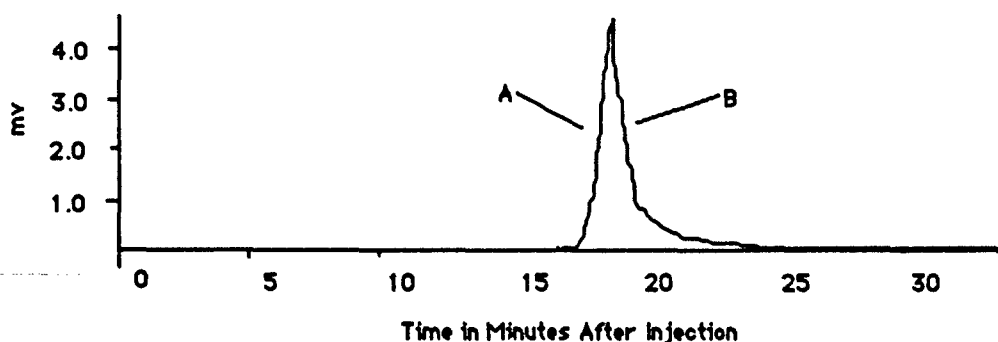


Figure 8. Semi-preparative HPLC chromatogram of ACN-insoluble material. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Peak eluant was separated at dotted line as fractions A and B. Detector set at 210 nm.

Chromatography of the ACN-insoluble material revealed a single peak which is a composite of several. These were split exactly at the top of the peak resulting in fractions A and B (Figure 8 above). The collected eluants were dried down and the fractions resuspended in 100  $\mu$ l of dry methanol. 10  $\mu$ l of these fractions were tested on the ileum preparation. Toxicity to the ileum was 100% for both peaks (Table 6, lines 6 & 7).

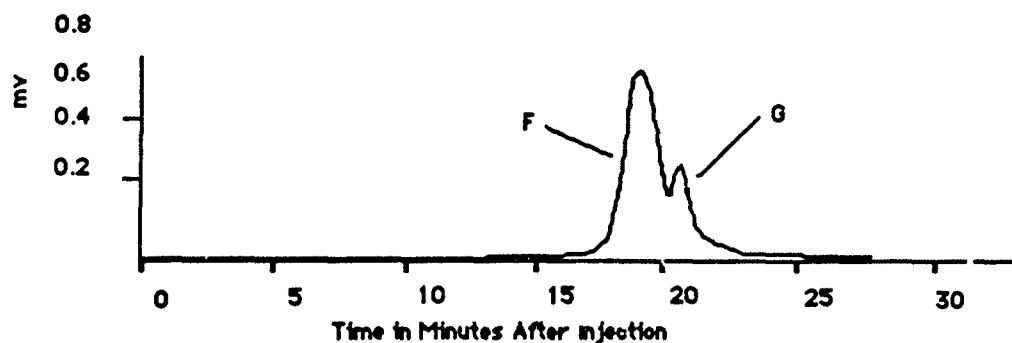


Figure 9. Semi-preparative HPLC chromatogram of fraction A from ACN-insoluble material isolated from water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

When fraction A was re-run on the semi-preparative HPLC two complexes were resolved, fractions F and G (Figure 9 above). G was relatively non-toxic, but F was toxic to the ileum (Table 6, lines 11 & 12).

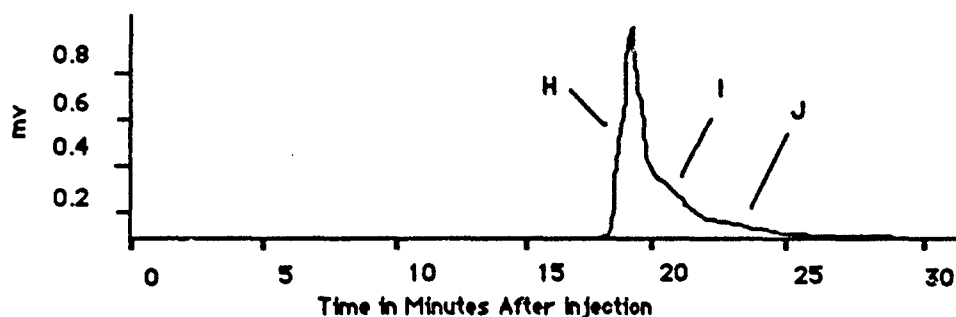


Figure 10. Semi-preparative HPLC chromatogram of fraction F from ACN-insoluble material isolated from water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

Re-chromatography of complex F resulted in a complex peak and the eluant was collected in three fractions, H, I and J (Figure 10 above). Of these three fractions collected only fraction I, the middle portion showed toxicity (Table 6, line 14).



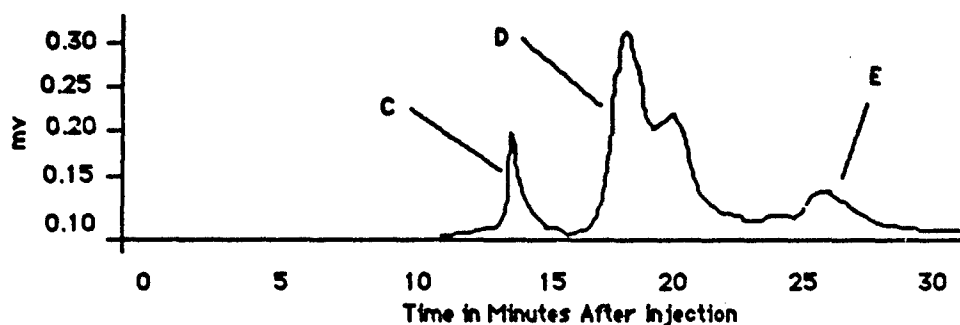


Figure 11. Semi-preparative HPLC chromatogram of the ACN-soluble material isolated from water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

The ACN-soluble fraction chromatographed into complex peaks C, D and E (Figure 11 above). When tested on the ileum preparation fraction C was toxic, D was slightly toxic and E was not toxic at all (Table 6, lines 8, 9 & 10).

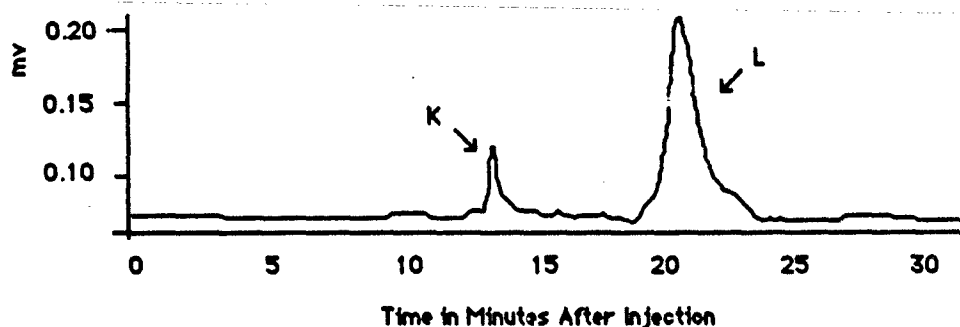


Figure 12. Semi-preparative HPLC chromatogram of fraction C from the ACN-soluble material isolated from water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

When fraction C was re-chromatographed (Figure 12 above) it resulted in a toxic peak K and a nontoxic peak L (Table 6, lines 16 & 17).

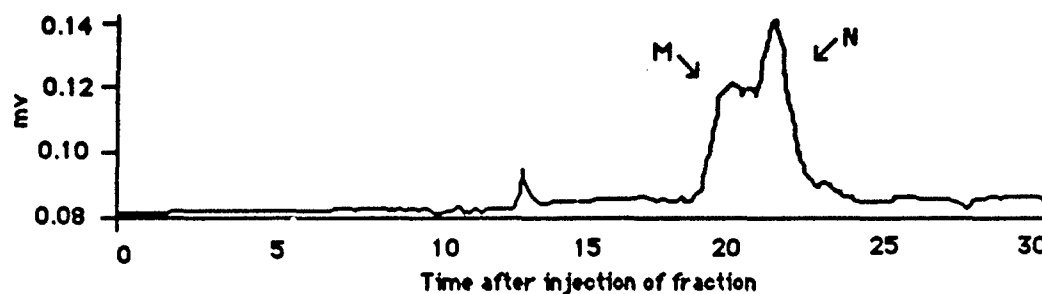


Figure 13. Semi-preparative HPLC chromatogram of fraction D from ACN-soluble material isolated from water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

When fraction D was re-chromatographed it separated into fractions M and N (Figure 13 above). Neither of these peaks were toxic to the ileum (Table 6, lines 18 & 19).

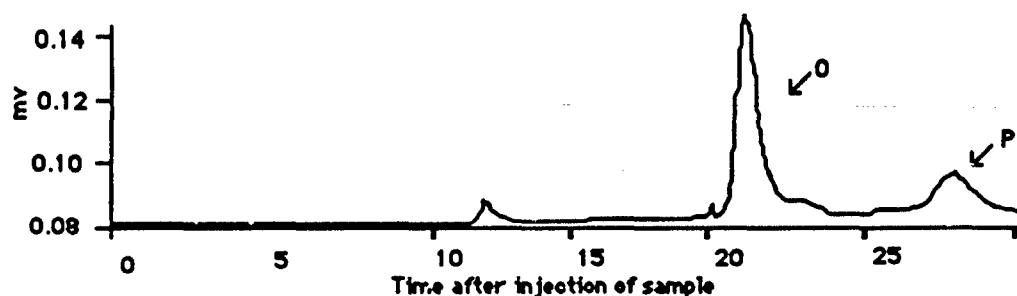


Figure 14. Semi-preparative HPLC chromatogram of fraction E from ACN-soluble material isolated from water soluble extract. Sample application was 50  $\mu$ l of a sample of 7 mg/ml. Running conditions were: 15 micron C-18 silica gel, 4.7 mm X 128 cm column, flow rate 0.2 ml/min, and 100% methanol as solvent. Detector set at 210 nm.

Re-chromatography of fraction E resolved it into peaks O and P (Figure 14 above). Again when tested on the ileum preparation neither of these fractions were toxic (Table 6 lines 20 & 21).

**TABLE 6**  
**ILEUM RESULTS OF HPLC FRACTIONS**

NO	SAMPLE	% INHIB		RESOLVED INTO PEAK
		@ 15 min	@ 90 min	
1	WS			
2	ACN-INS	100	100	
3	ACN-SOL-1	100	100	
4	ACN-SOL-2	0	0	
5	ACN-SOL-3	0	0	
6	A	100	47	F & G
7	B	100	100	H, I & J
8	C	97	94	K & L
9	D	34	26	M & N
10	E	0	1	O & P
11	F	98	100	
12	G	18	14	
13	H	0	0	
14	I	98	100	
15	J	0	0	
16	K	95	68	
17	L	0	0	
18	M	0	0	
19	N	3	0	
20	O	9	0	
21	P	0	0	

Above test were conducted on ilea by injecting 10  $\mu$ l directly into the bath. The % inhibition listed at 15 and 90 min are as % of the acetylcholine control. WS = water soluble extract. ACN-INS = acetylcholine insoluble material. ACN-SOL = acetylcholine soluble material. Letters A through P represent HPLC fractions which were collected and tested for toxicity.

After these preliminary runs on the semi-preparative system were completed, we then took one ml of water soluble material (7 mg/ml) and subjected it to ACN extraction. The resultant ACN-soluble and ACN-insoluble products were blown dry and resuspended in 1 ml of dry methanol. First the entire one ml of the ACN-insoluble material was separated on the preparative column. The column eluants were collected between 10 and 50 minutes after ACN-insoluble injection in 50 ml samples (Figure 15 next page).

Twenty seven 50 ml samples were collected resulting in a total of 1350 ml. Each of the 27 samples were lyophilized down to a volume of approximately 2 ml and 10  $\mu$ l aliquots taken for testing on the guinea pig ileum. Once the ileum aliquoting was completed the samples were blown completely dry and weighed on the Cahn microbalance in a tared vessel. All the samples were then

reconstituted to 1 ml with dry methanol and an analytical run completed on ten  $\mu$ l of material. This procedure was utilized in order not to miss a minor toxic component which may be present in very small sample amounts and be lost in diluting up from small volumes.

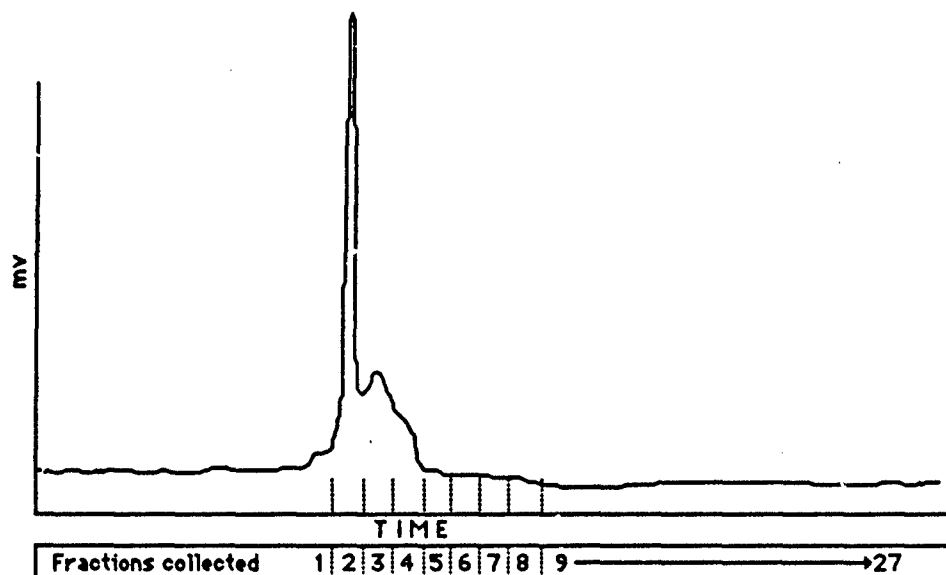


Figure 15. Preparative HPLC chromatogram of ACN-soluble material isolated from water soluble extract. Sample application was 1 ml of a sample of 7 mg/ml. Sample loaded on column in 4 min using flow rate of 10 ml/min. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm.

We are presently about 50% finished with the ileum runs on the ACN-insoluble material. The preparative separation of the ACN-soluble material and untreated water soluble extract has been completed. Samples from both of these have been taken for the ileum assay. This experiment with the preparative column was designed to demonstrate the correspondence of chromatograms when switching from semi-preparative to preparative systems, the precise localization of toxic components coming off the column and a preliminary to running larger amounts on the preparative column. It is important to note at this point that these three runs (Crude, ACN-soluble and ACN-insoluble) on the preparative HPLC produced over 100, one ml fractions. Each one of these fractions were tested on the ileum preparation for toxicity using only 10  $\mu$ l of each fraction. The savings in terms of mice and toxin is a significantly large amount.

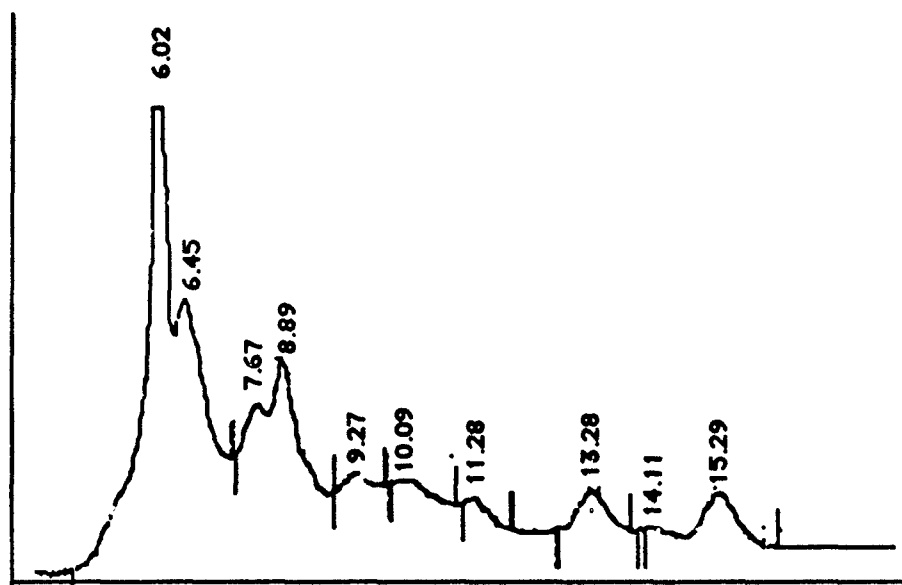


Figure 16. Analytical HPLC chromatogram of water soluble extract. Sample application was 15  $\mu$ l of a sample of 3.59 mg/ml. Peak elution times are displayed above the peaks detected. Running conditions were 10 micron C-18 RCM column. Flow rate 0.2 ml/min and 100% methanol as solvent.

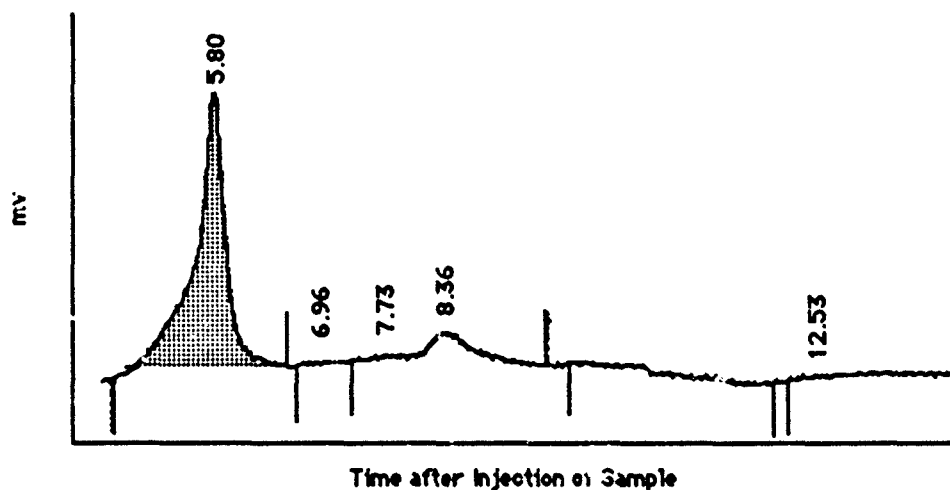


Figure 17. Analytical HPLC chromatogram of "maitotoxin" peak collected directly from repetitive analytical runs. Sample application was 10  $\mu$ l of a sample of 400  $\mu$ l. Peak elution times are displayed above the peaks detected. Running conditions were 10 micron C-18 RCM column. Flow rate 0.2 ml/min and 100% methanol as solvent. Shaded area of chromatogram is toxic area.

In accordance with our previously published method for HPLC purification of "maitotoxin" (46), We have been using the analytical HPLC to collect individual peaks for accumulation and delivery, if so desired. Thus far, we have accumulated 700  $\mu$ l of the initial peak. This perhaps represents a total of 2  $\mu$ g of toxin. The relative purity can be estimated by comparing an HPLC chromatogram of the crude material (Figure 16) with one of the isolated material (Figure 17).

•NMR DATA As stated in our last report we examined five model compounds. These compounds were chosen for their biomedical interest, because they had structures similar to what we are expecting in our isolated toxins and also to demonstrate our NMR capabilities. The five compounds chosen were: atropine, quinine, procaine, xanthosine dihydrate and reserpine.

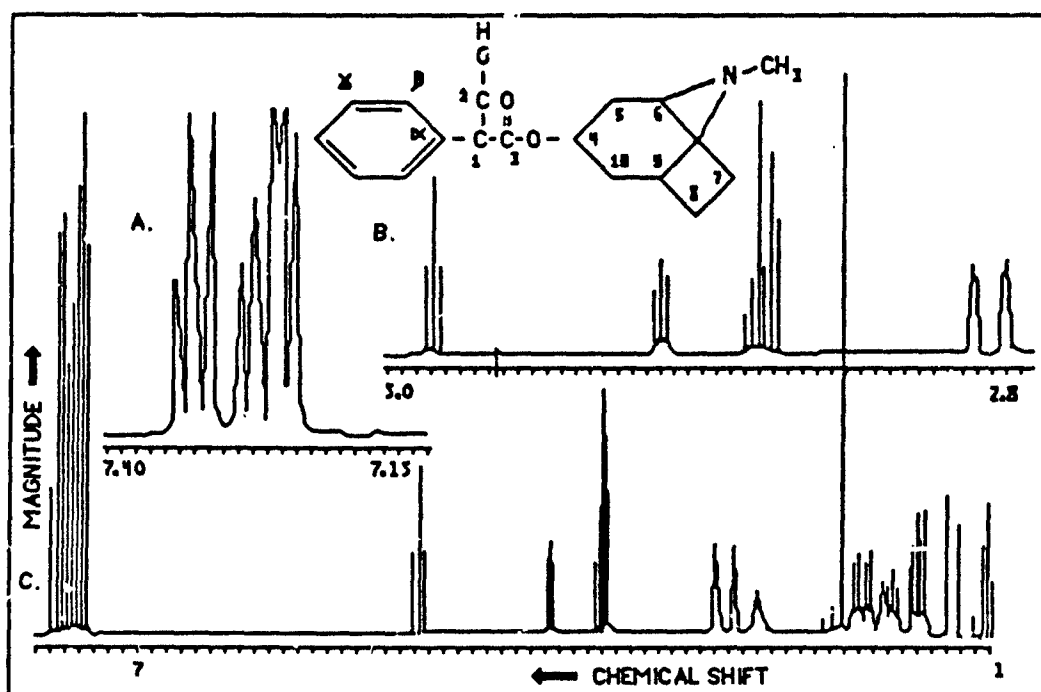


Figure 18. Proton coupled 500 MHz spectra of atropine. Upper insert right shows the 2.8 to 5.0 region.

Atropine. The F-T high resolution proton spectrum of atropine,  $C_{17}H_{23}NO_3$  in deuteriochloroform,  $CDCl_3$ , is shown in Figure 18. Note the distinct resolution of each peak. The upper insert shows an

enlargement of the 2.8-5.0 PPM spectral region. The resonance assignments are made by location in the appropriate chemical shift range and by selective decoupling double resonance experiments. We show here  $^1\text{H}$  assignments and accompanying coupling constants.

# $^1\text{H}$ PROTON ASSIGNMENTS

RingM	C1H	C2HA	C2HB	C4H	C5HA	C5HB	NH <sup>2</sup>	C10HA	C10HB	C6H	C7HA
7.3	4.14	3.8	3.75	5.0	1.99	1.42	2.17	2.07	1.63	2.87	1.72
				</							

## $^1\text{H}$ - $^1\text{H}$ COUPLING CONSTANTS DETERMINED BY DOUBLE RESONANCE

J1,2A	J1,2B	J2A,2B	J4,5A	J4,5B	J4,10A	J4,10B	J5A,5B	J5A,6
9.0	4.6	2.0	5.0	0	5.1	0	15.0	3.5
J5A,7A	J6,7A	J7A,7B	J8A,8B	J8A,9	J8A,10A	J9,10A	J10A,10B	
1.2	7.0	8.4	7.3	7.0	1.2	3.5	15.0	

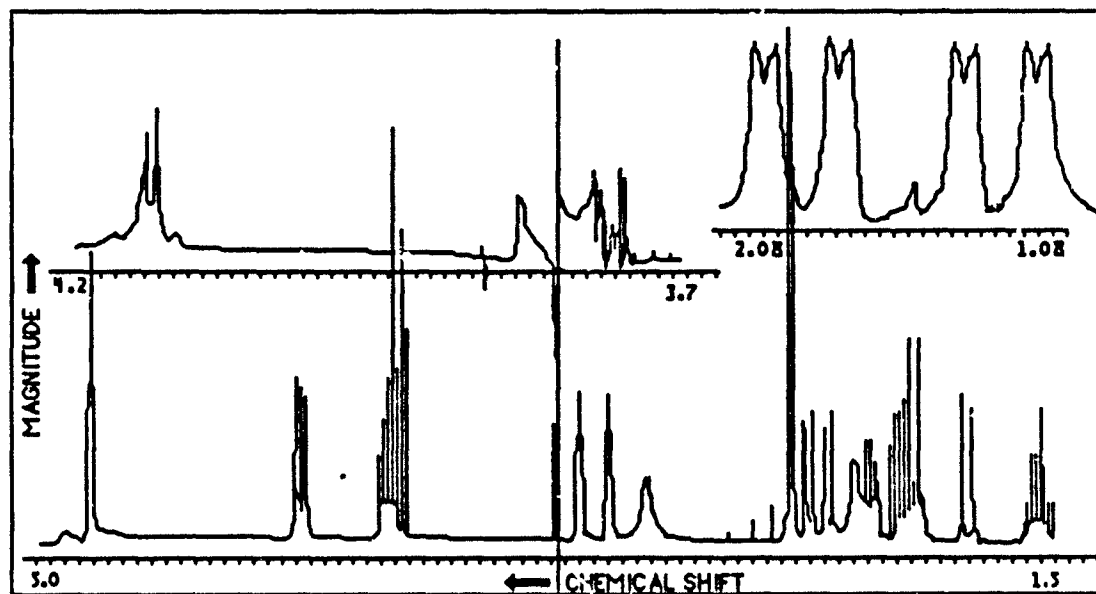


Figure 19. Proton coupled 500 MHz spectra of atropine. 1.4 to 5.2 PPM region of proton spectra with 3.80 (C2HA) decoupled. Upper left region is 3.7 to 4.2 PPM region. Upper right inset shows multiplets at 2.07 (C10HA) and 1.99 (C5HA).

As is characteristic in rigid systems, the methylene protons resolve individually and we designate the low field proton as A and the up field proton as B. Figure 19 shows the decoupling of C2HA (3.80) and the resulting 4.6 Hz doublet at 4.15 (C1H). This 4.6 Hz splitting is the coupling constant between C1H and C2HB. The upper right insert of Figure 18 shows the resulting multiplets at 2.07 (C10HA) and 1.99 (C5HA) after decoupling C4H at 5.0. Here coupling constants of 15.0, 3.7, and 1.2 Hz can be seen. A coupling constant of 5.5 Hz is thereby lost in the decoupling of C4H from both C10HA and C5HA. This further shows the symmetrical nature of the saturated ring structure of this molecule.

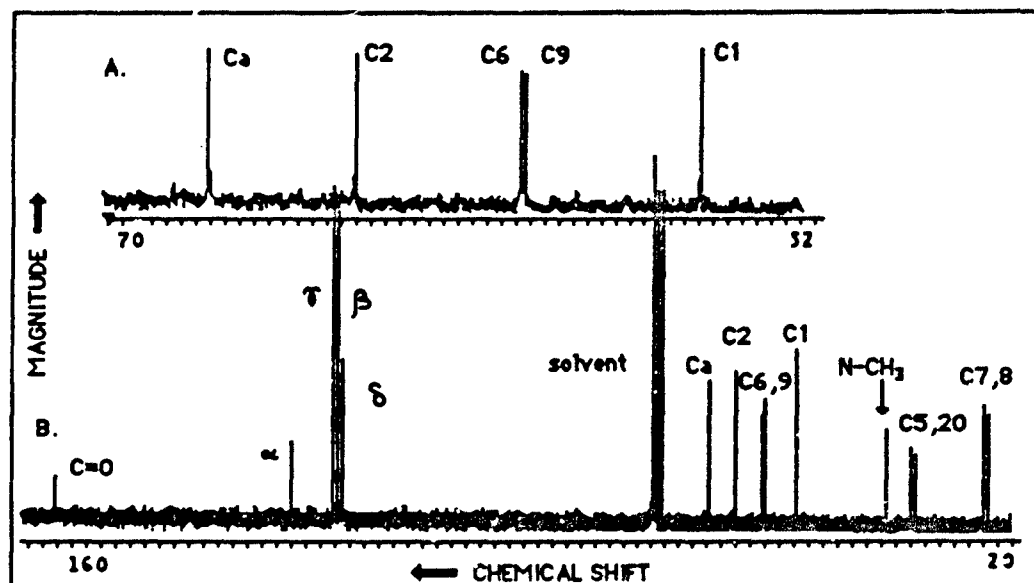


Figure 20. 300 MHz  $^{13}\text{C}$  NMR spectra of atropine.



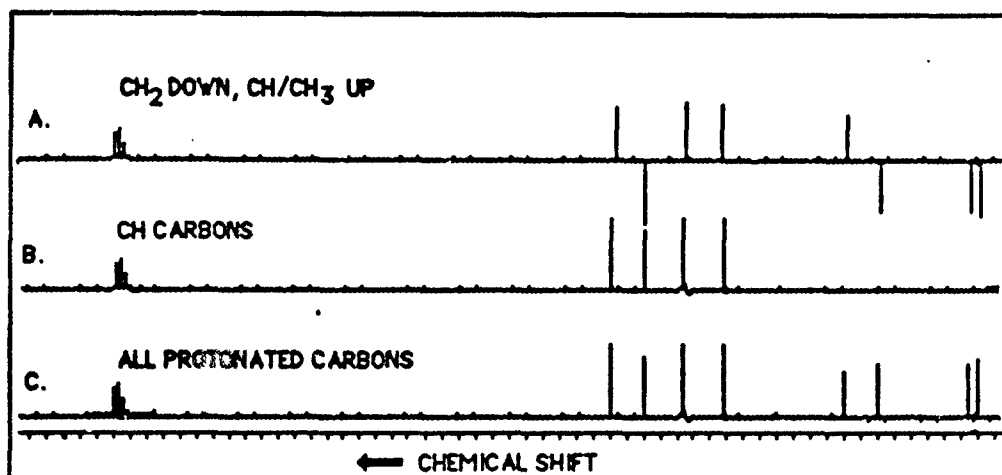


Figure 21. Distortionless, enhancement polarization transfer plot of atropine. This plot differentiates between CH, CH<sub>2</sub> and CH<sub>3</sub> carbons.

The proton decoupled <sup>13</sup>C spectrum is shown in Figure 20. Each carbon resolved separately with the exception of the double resonance of α and β carbons. This situation is easily recognized by the approximate doubling of height of the spectral line. The insert at the top of Figure 20 shows an enlargement of the region from 52 to 70 PPM, thus allowing the two distinct lines at 59.5 to be clearly visible.

Figure 21 is a spectral plot of carbon-proton correlation called a DEPT plot, short for Distortionless Enhancement Polarization Transfer. This plot shows all protonated carbons and distinguishes between CH, CH<sub>2</sub>, and CH<sub>3</sub> carbons. Quarternary carbons do not register on DEPT but can be distinguished from the normal proton decoupled <sup>13</sup>C spectra as shown in Figure 20. For atropine here, the carbonyl (172 PPM) and ring alpha carbon (136 PPM) are seen to be quarternary carbons. The resonance line at 64 PPM shows on DEPT as CH<sub>2</sub> and hence must be the hydroxyl carbon, C2, being in the 60-80 PPM range. Assignment of the methyl carbon at 40.2 PPM would be difficult without DEPT which makes it unambiguous. Hence, the value of this plot is most obvious.

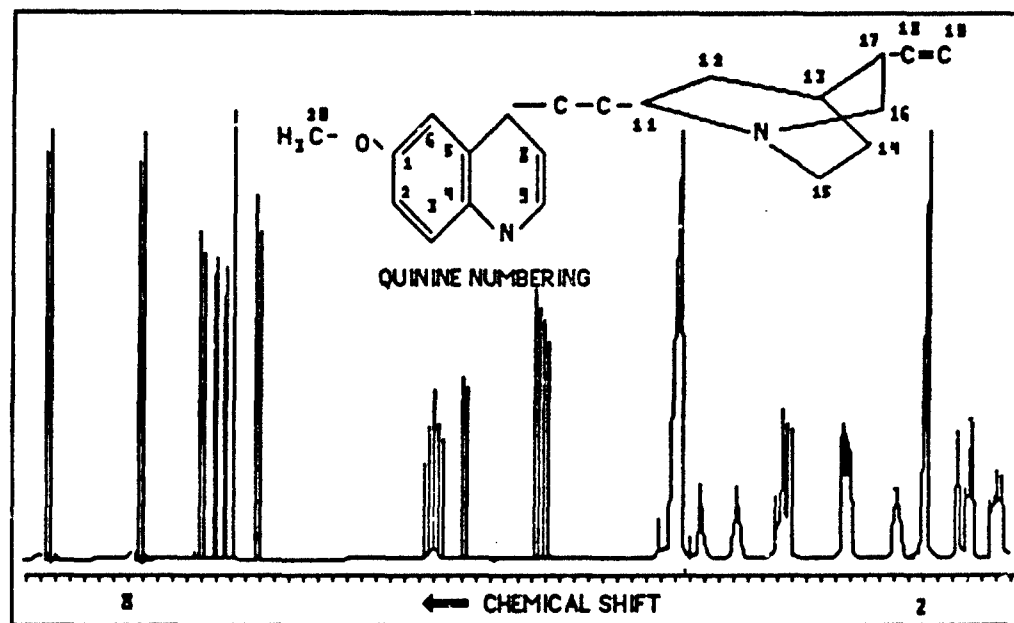


Figure 22. Proton coupled 500 MHz spectra of quinine. 1.3 to 8.6 spectrum with  $^1\text{H}$  assignments.

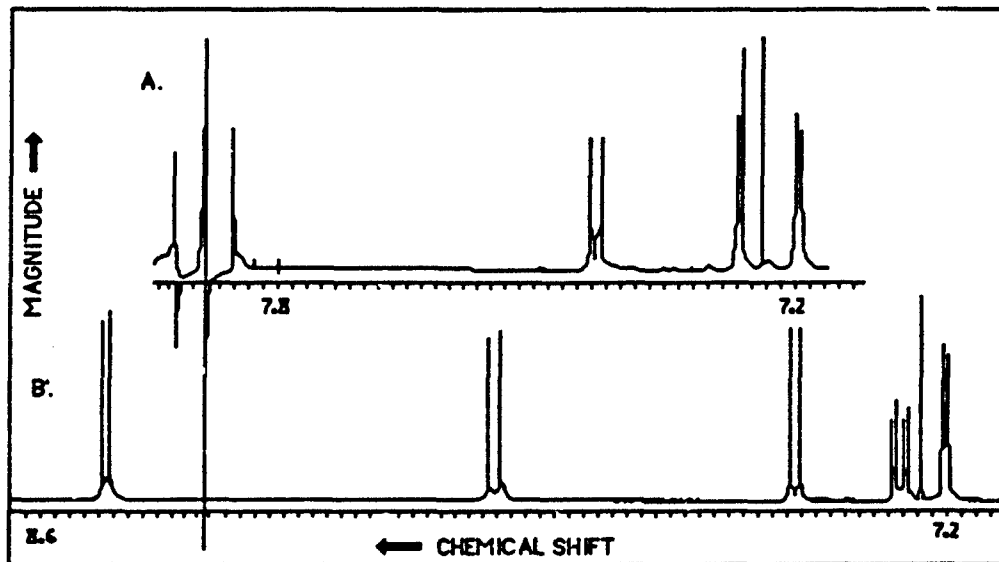


Figure 23. Proton coupled 500 MHz spectra of quinine in the region of 7.0 to 8.6 PPM. Upper insert shows the low field region with  $d$ -coupling of C3H from C2H.

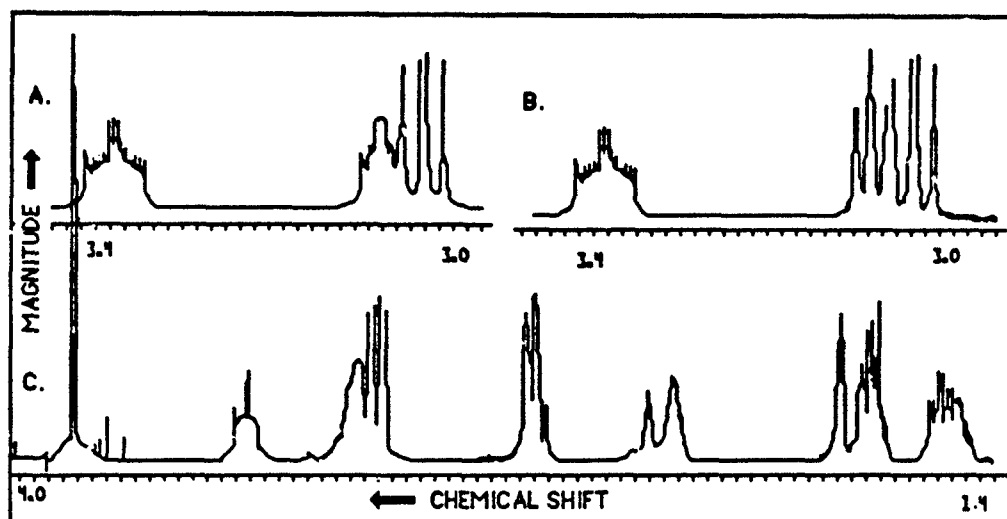


Figure 24. Proton coupled 500 MHz spectra of quinine. 1.3 to 4.0 spectrum with  $^1\text{H}$  assignments. Upper inserts show resulting spectra before and after decoupling  $\text{C}10\text{H}$ .

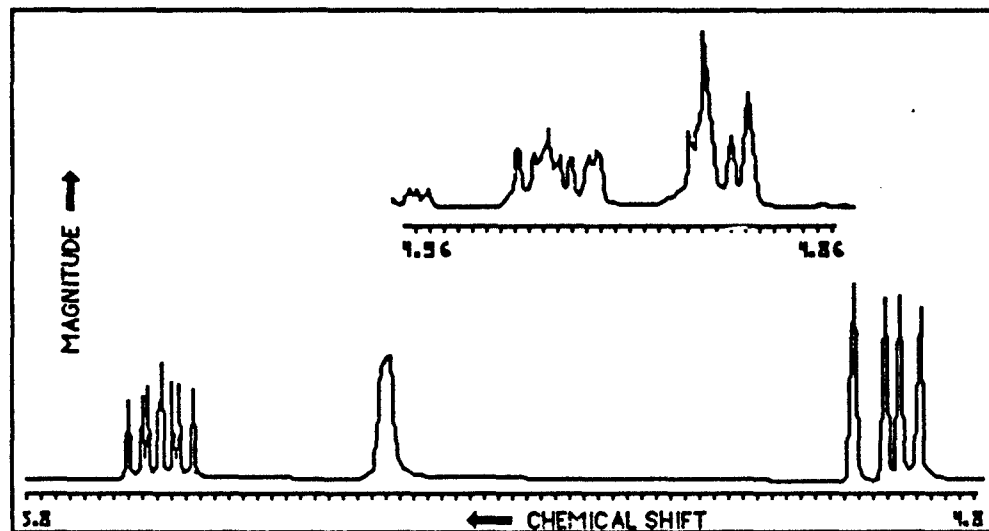


Figure 25. Proton coupled 500 MHz spectra of quinine. Upper insert shows resulting spectra with  $\text{C}18\text{H}$  decoupled.

Quinine,  $\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2$  has several interesting features; a fused benzene ring with a N containing ring, a rigid bridged structure containing N, and a terminal olefinic group. The NMR data for

quinine are shown in Figures 22 to 28. The  $^1\text{H}$  spectral assignments are shown as determined by double resonance with the accompanying coupling constants. The large coupling constants of the saturated ring is characteristic of these rigid structures.

#### $^1\text{H}$ PROTON ASSIGNMENTS

C9H	C3H	C8H	C2H	C6H	C18H	C10H	C19H(trans)	C19(cis)	C20H	C10(OH)	
C15HA											
8.60	7.92	7.48	7.28	7.20	5.71	5.50	4.93	4.88	3.88	3.68	3.40
C11H	C16HA	C15HB	C16HB	C16H	C13H	C14HA	C12HA	C12HB	C14HB		
3.08	3.03	2.62	2.60	2.22	1.78	1.70	1.68	1.51	1.46		

#### $^1\text{H}$ - $^1\text{H}$ COUPLING CONSTANTS DETERMINED BY DOUBLE RESONANCE

J2,3	J2,6	J8,9	J10,11	J11,12A	J11,12B	J12A,12B	J17,16A	J16A,16B		
9.2	2.7	4.7	4.1	9.2	9.2	11.7	10.3	13.04		
J15A,15B	J14A,14B	J18,19a	J18,19c	J19a,19c	J13,18	J13,17	J17,18	J17,19c		
11.1	14.5	17.2	10.4	1.8	1.6	8.9	7.0	6.0		

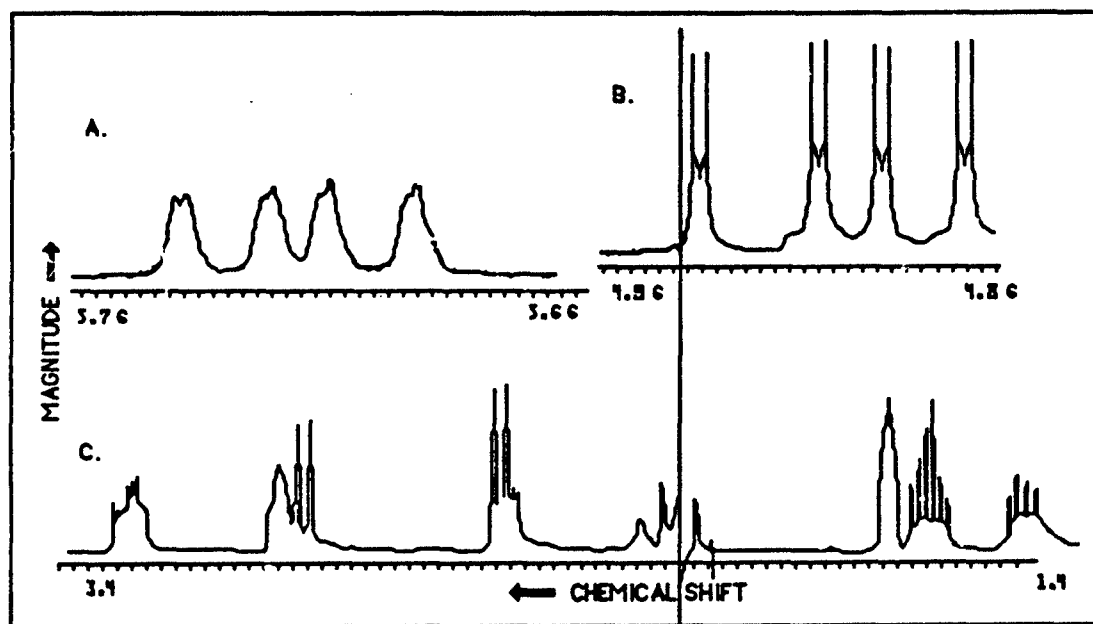


Figure 26. Proton coupled 500 MHz spectra of quinine. Inserts show resulting spectra with C17H decoupled.

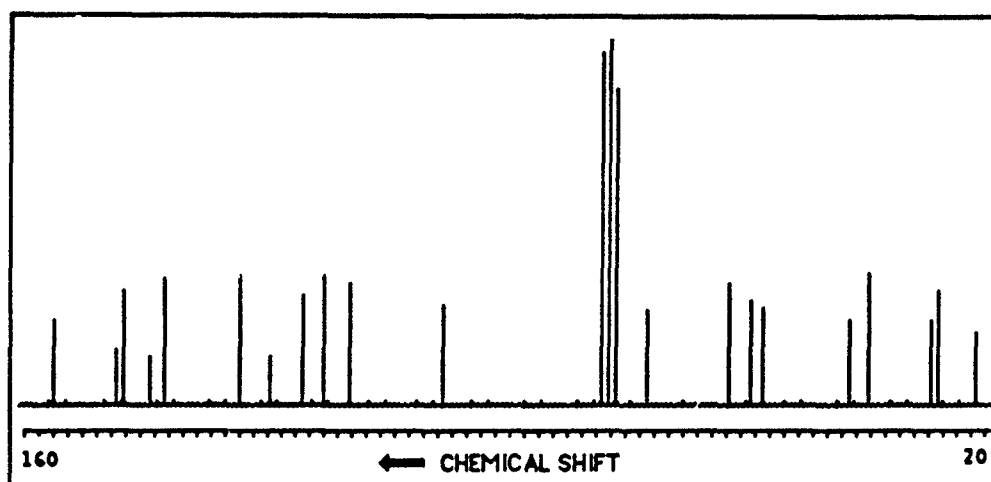


Figure 27. 300 MHz  $^{13}\text{C}$  spectrum of quinine.

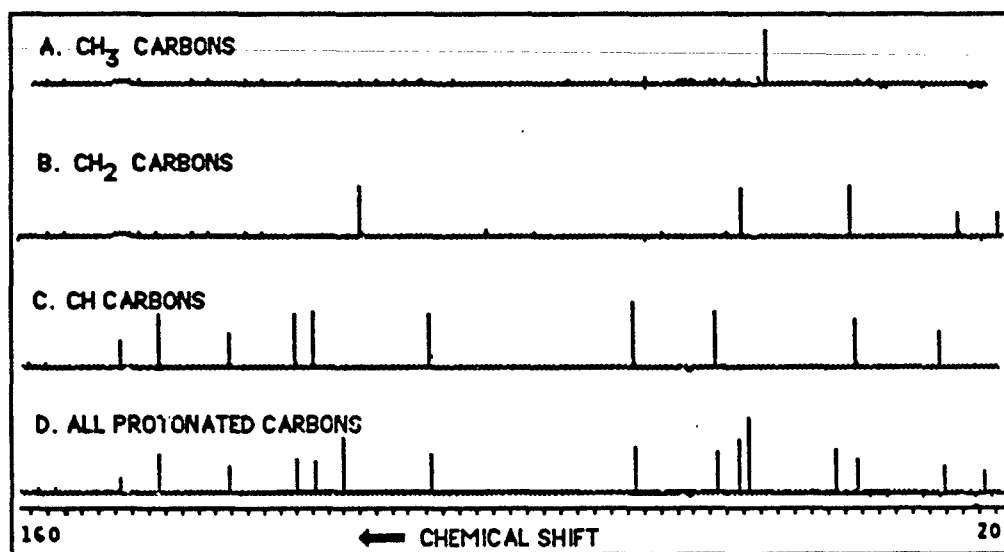


Figure 28. Distortionless, enhancement polarization transfer plot of quinine.

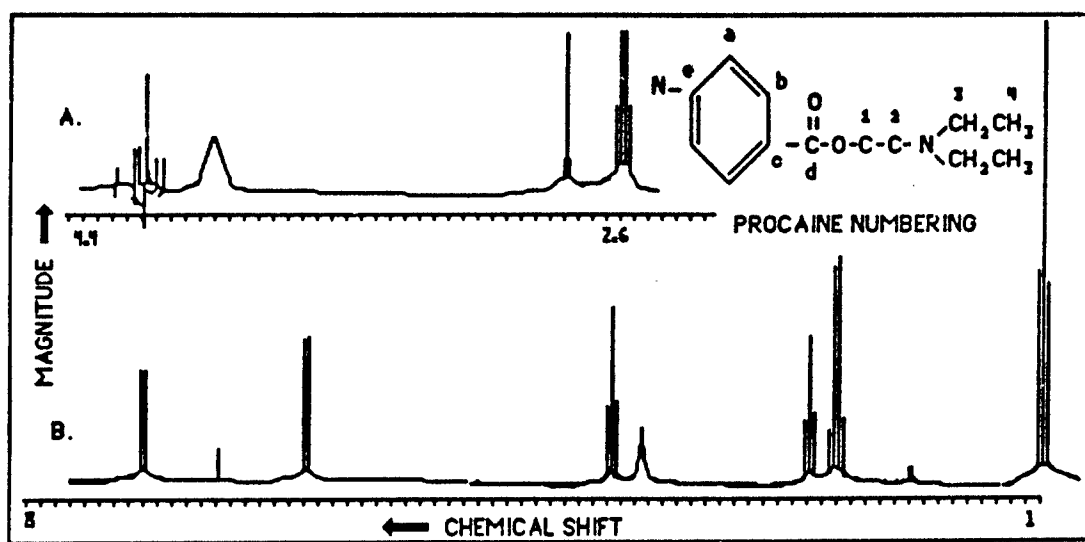


Figure 29. Proton coupled 500 MHz spectra of procaine.

Procaine is a relatively simple molecule as analyzed by NMR but provides valuable information concerning the chemical shifts of protons on carbon bonded to nitrogen. As this molecule is not a rigid structure like other molecules described in this report, its NMR analysis is particularly simple. As shown in the proton spectrum, Figure 29, the benzene ring is completely symmetrical with the two protons  $\alpha$  to N resolving at lowest field, 7.8 PPM while the two protons  $\beta$  to N resolve at 6.6 PPM. Vicinal couplings of 8.7 Hz characterize the splitting. The geminal protons of the chain resolve together and show first order couplings. The upper insert in Figure 29 shows the decoupling of the C1H protons (4.30) from the C2H protons (2.8), where the 6.3 Hz triplet at 2.80 PPM collapses to a singlet.

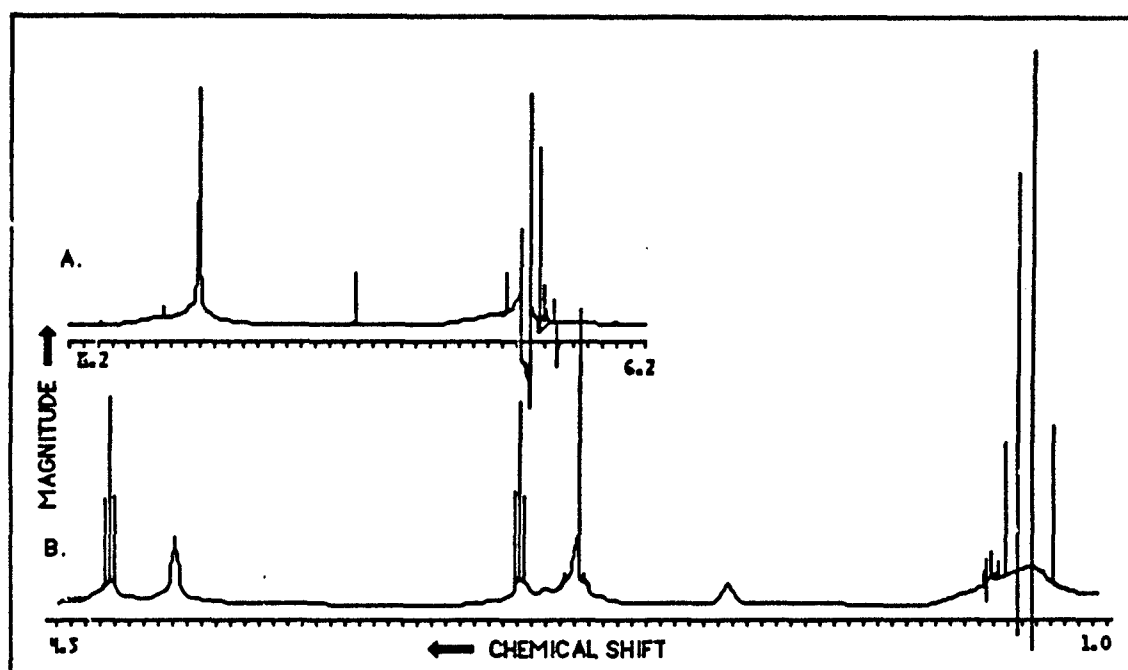


Figure 30. Proton coupled 500 MHz spectra of procaine with decoupling of terminal methyl carbon.

Figure 30 shows decoupling of the terminal methyl protons (1.0 PPM) with resulting collapse of the C3H methylene protons to a singlet at 2.60. The upper insert here shows the decoupling of the ring proton. The resonance at 4.05 PPM must be the NH protons, present in all sample observed and integrating fully to two protons.

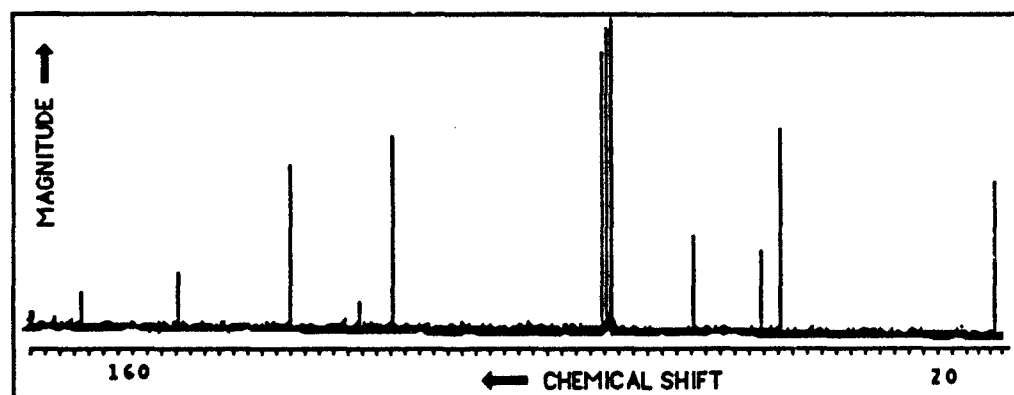


Figure 31. 300 MHz  $^{13}\text{C}$  NMR spectrum of procaine.

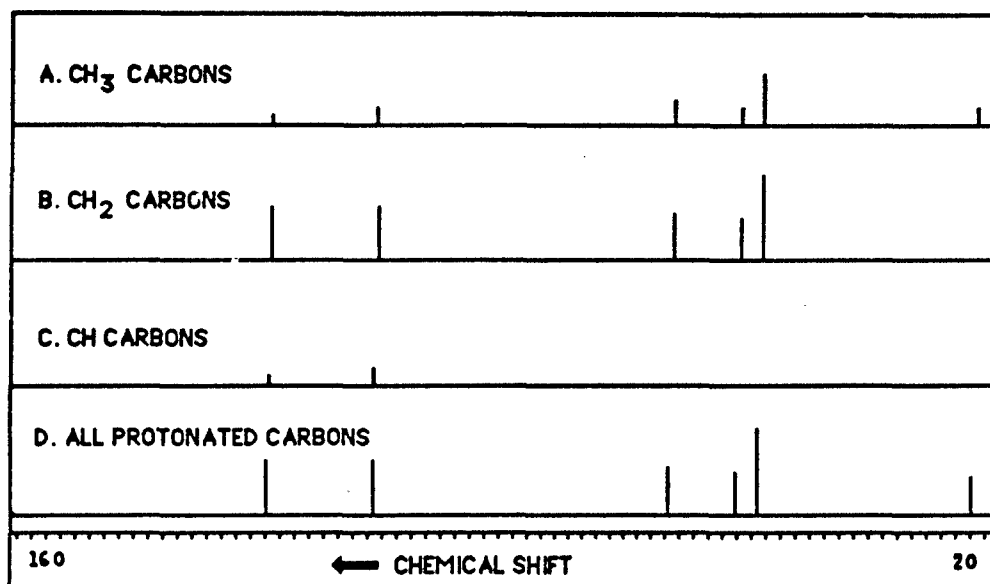


Figure 32. Distortionless, enhancement polarization transfer plot of procaine.

The proton decoupled  $^{13}\text{C}$  spectra is shown in Figure 31. Quarternary carbons are observed at 166P, C=O; 151 P, the ring carbon  $\alpha$  to N, and 120 P, the ring carbon bonded to the carbonyl carbon. The height of the resonance lines at 132 P and 114 P indicate 2 carbons each, even in light of somewhat attenuated heights of quarternary carbon resonance lines. This 2 to 1 ratio is also seen for the branched  $\text{CH}_2$  and  $\text{CH}_3$  resonances in the chain. A DEPT plot is also shown for completeness in Figure 32.



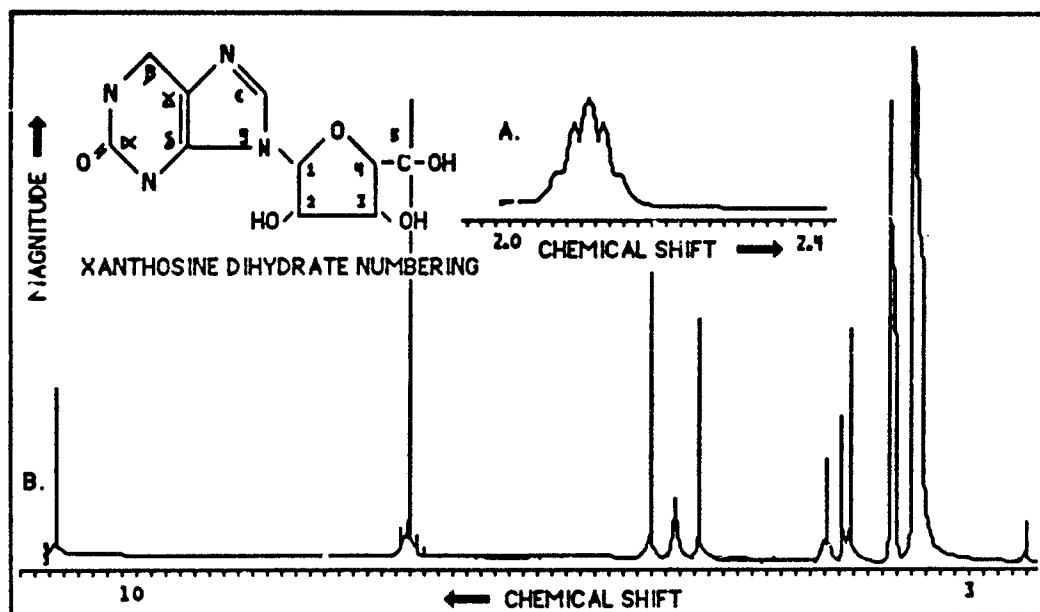


Figure 33. Proton coupled 500 MHz spectra of xanthosine dihydrate.

Xanthosine dihydrate. Due to limited solubility of this compound in conventional solvents,  $\text{CDCl}_3$ ,  $\text{CD}_3\text{OD}$ ,  $\text{CD}_3\text{CN}$ ,  $\text{D}_2\text{O}$ , etc., we have examined xanthosine dihydrate in dimethylsulphoxide (DMSO). We were very pleased to find that the resonance due to hydroxal protons could be observed, a phenomena not normally seen when using proton donating solvents.

The full proton spectrum in DMSO is shown in Figure 33. The singlets at lowest field 10.85 and 7.85 must be ring N-H and C-H protons respectively and the lowest field midrange resonance at 5.72 must be the proton on the ribose ring carbon bonded to O and N, designated C1H. We then used selective decoupling to determine the remaining assignments.

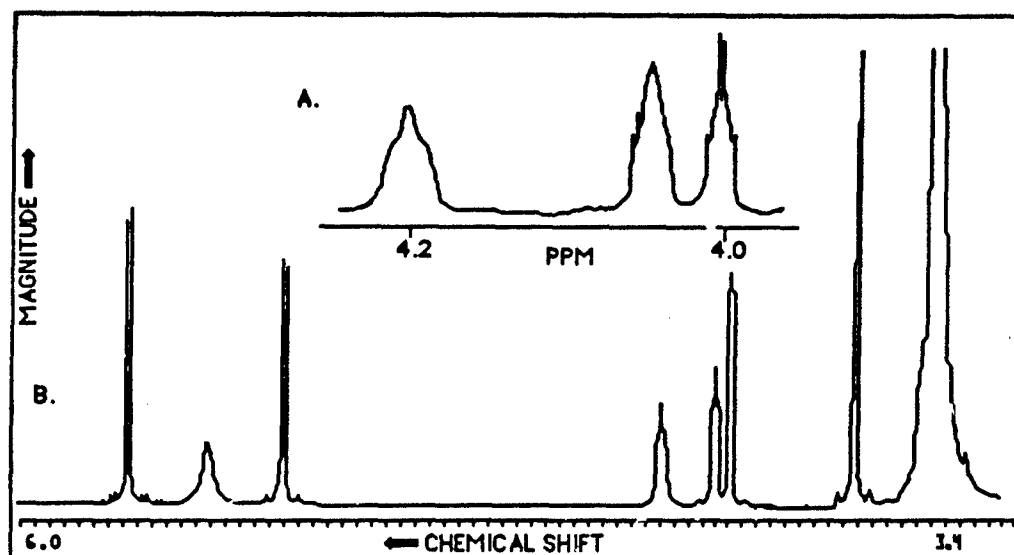


Figure 34. Proton coupled 500 MHz spectra of xanthosine dihydrate. 6.0 to 3.0 PPM region of proton spectra

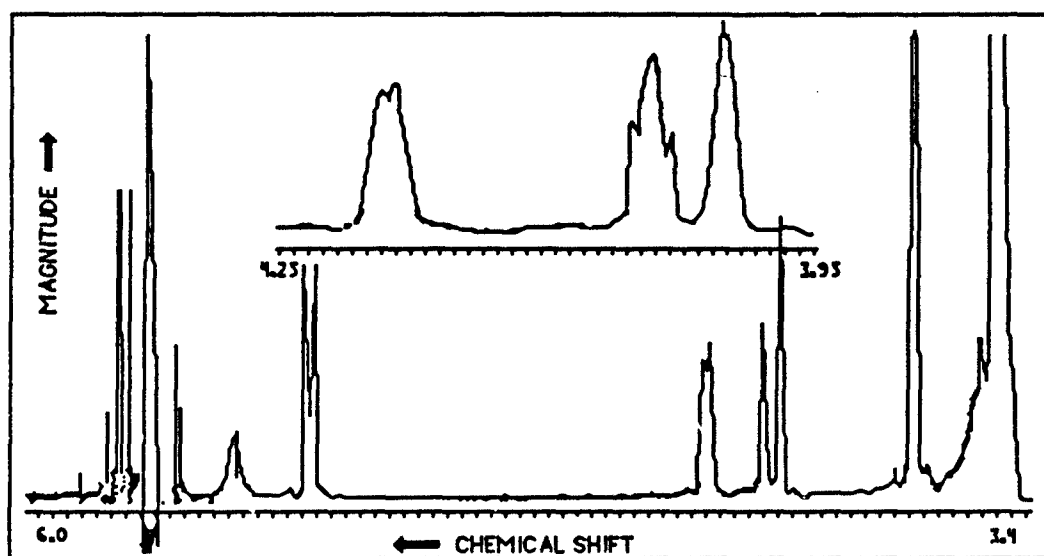


Figure 35. Proton decoupled 500 MHz spectra of xanthosine dihydrate. 3.0 to 6.0 region of spectrum with 5.72 (C1H) decoupled.

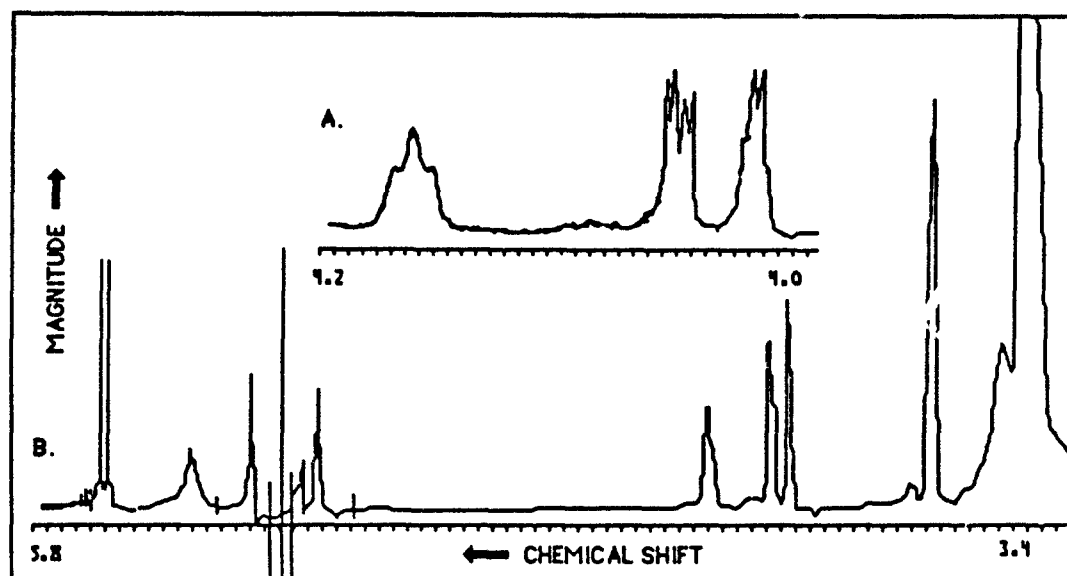


Figure 36. Proton decoupled 500 MHz spectra of xanthosine dihydrate.

In Figure 34 the proton of spectra from 6.0-3.0 PPM is shown revealing 7 peaks, excluding the large OH peak at 3.4 due to dihydrate and NaOH inherent in the compound. The upper insert, Figure 34A shows the fine structures of the 4.0 PPM peaks. Decoupling of 5.72 (C1H) and collapse of 4.20 (C2H) is shown in Figure 35, a 4.9 Hz doublet being observed at 4.20. Note that this is the only peak affected thereby assigning the 4.20 resonance as C2H. In Figure 36, the 5.29 resonance is decoupled, collapsing the 4.04 peak (C3H) into a split doublet, 5.0 and 2.1 Hz, thus establishing the coupling between 4.21 (C2H) and 4.05 (C3H) through the 4.9 coupling constant (Figure 35 and Figure 37).

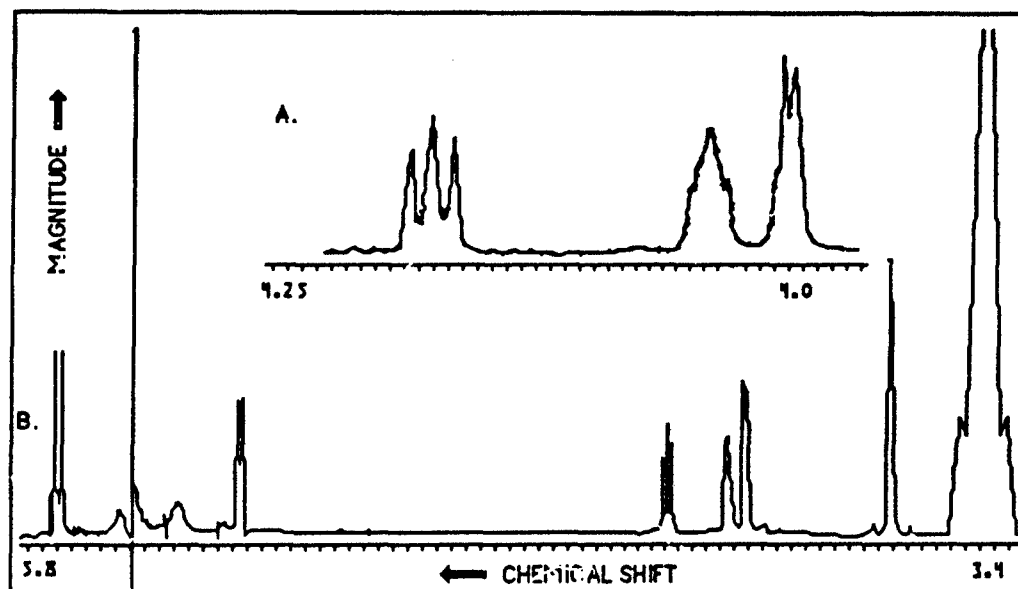


Figure 37. Proton decoupled 500 MHz spectra of xanthosine dehydrate with OH decoupled.

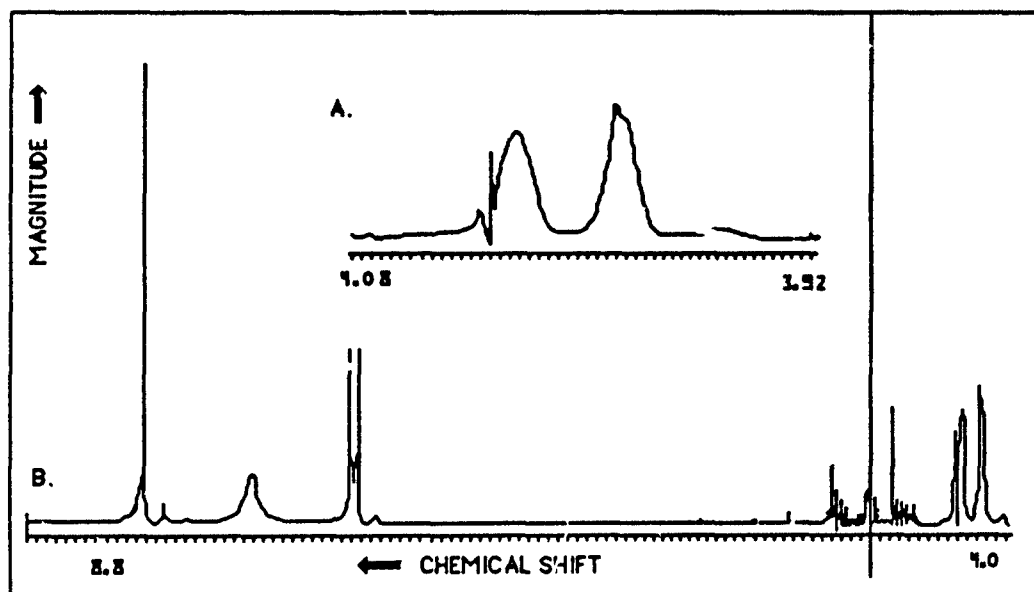


Figure 38. Proton decoupled 500 MHz spectra of xanthosine dihydrate.

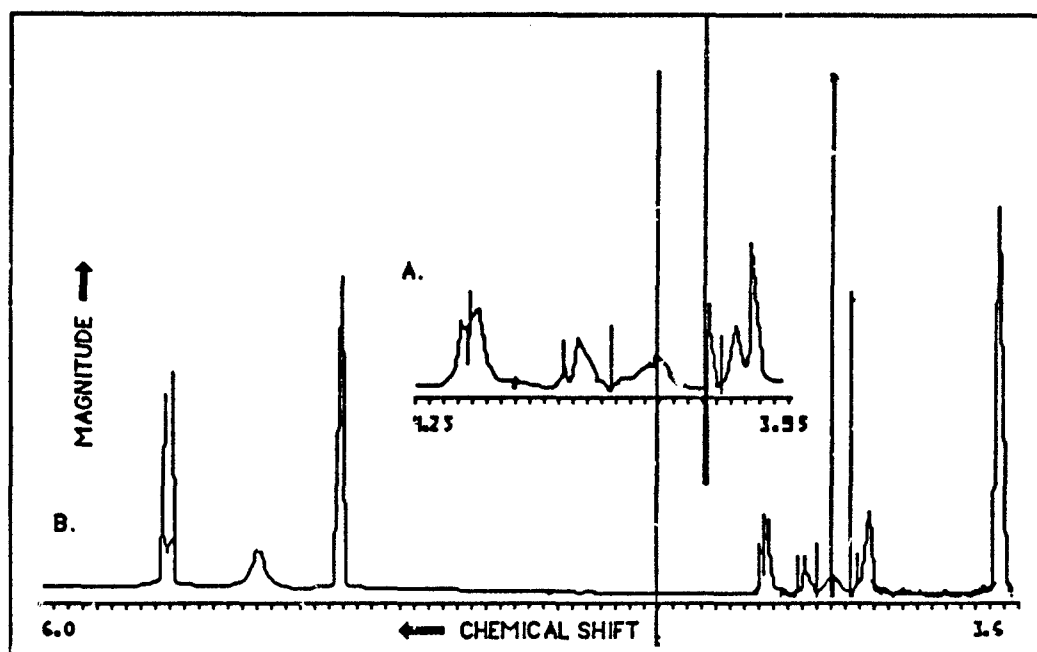


Figure 39. Proton decoupled 500 MHz spectra of xanthosine dihydrate.

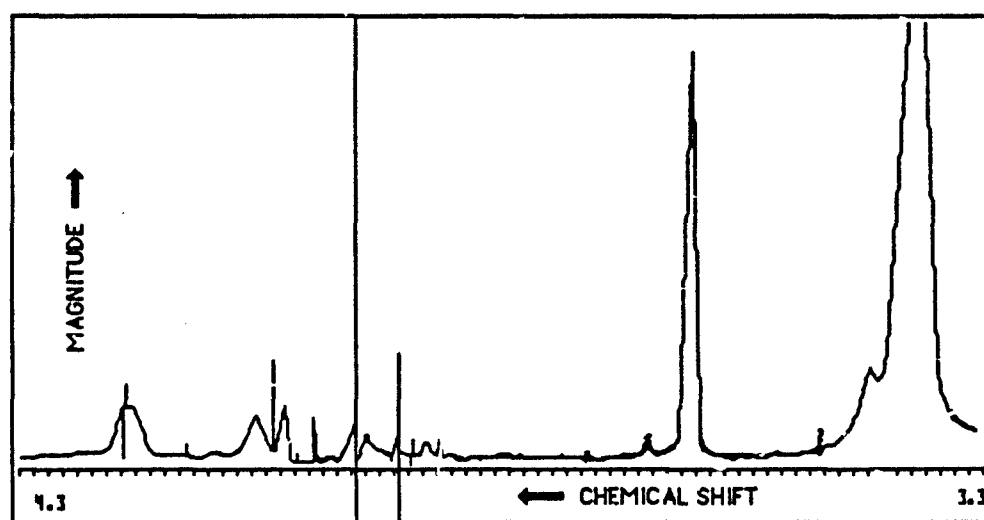


Figure 40. Proton decoupled 500 MHz spectra of xanthosine dihydrate. Decoupling at 3.98 PPM.

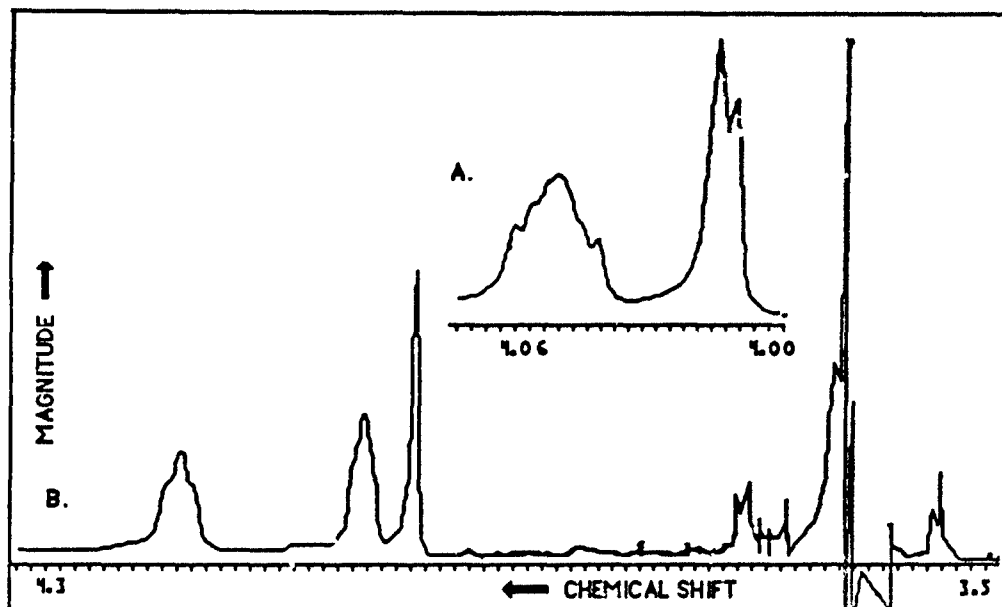


Figure 41. Proton decoupled 500 MHz spectrum of xanthosine dihydrate.

When the broad OH peak is decoupled as shown in Figure 37, the distinct splitting of 4.20 is seen as actually four lines of coupling constants 6.9 Hz and 4.9 Hz. These are the couplings of C2H with C1H and C3H respectively. In DMSO a small coupling of C2H and C2OH must therefore exist, thus blurring 4.20 in the undecoupled spectra. Figure 38 shows the decoupling of 4.20 (C2H) and the resulting singlet at 5.72 (C1H).

Figure 39 shows the decoupling of 4.05 (C3H) and resulting 7.0 doublet (C2H) at 4.20 and singlet at 5.29 (C4H). Decoupling the 3.98 resonance as shown in Figure 40 collapses 3.63 to a singlet. As this resonance integrated to 2 protons and is in the 3.6-3.8 range, the methylene of CH<sub>2</sub>OH, it is assigned as C5H<sub>2</sub>, and the 4.0 resonance as C3, C5OH, hydroxyl resonances. When 3.63 is decoupled as shown in Figure 41, the side bands of the 4.0 resonances are lost, with 2 Hz splitting remaining, indicating coupling between C3H and C3OH.

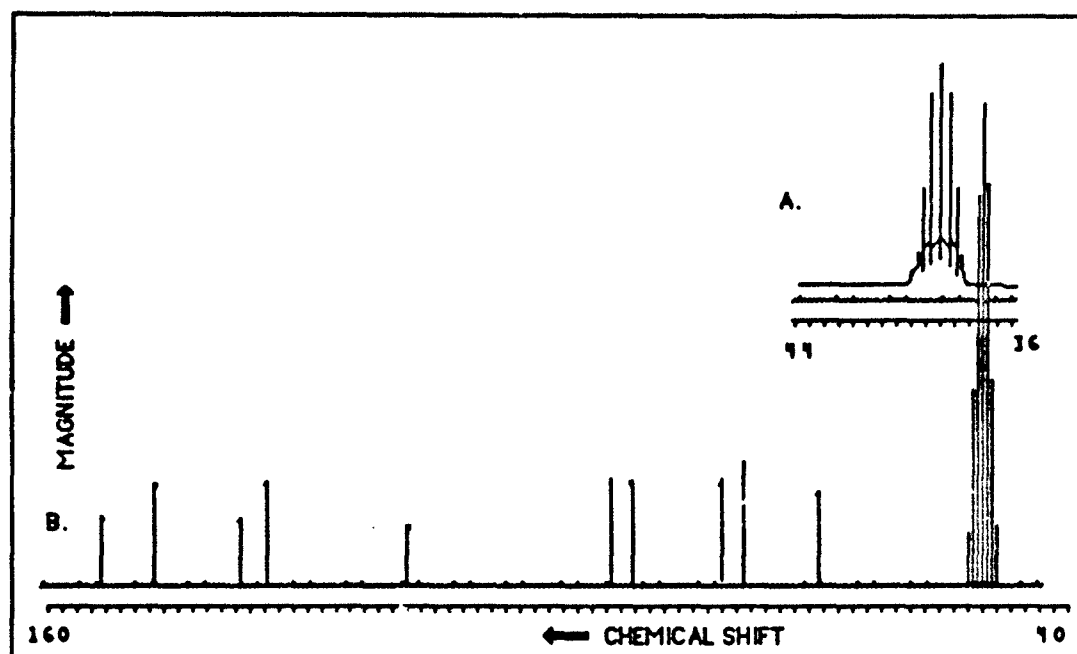


Figure 42.  $^{13}\text{C}$  spectrum of xanthosine dihydrate.

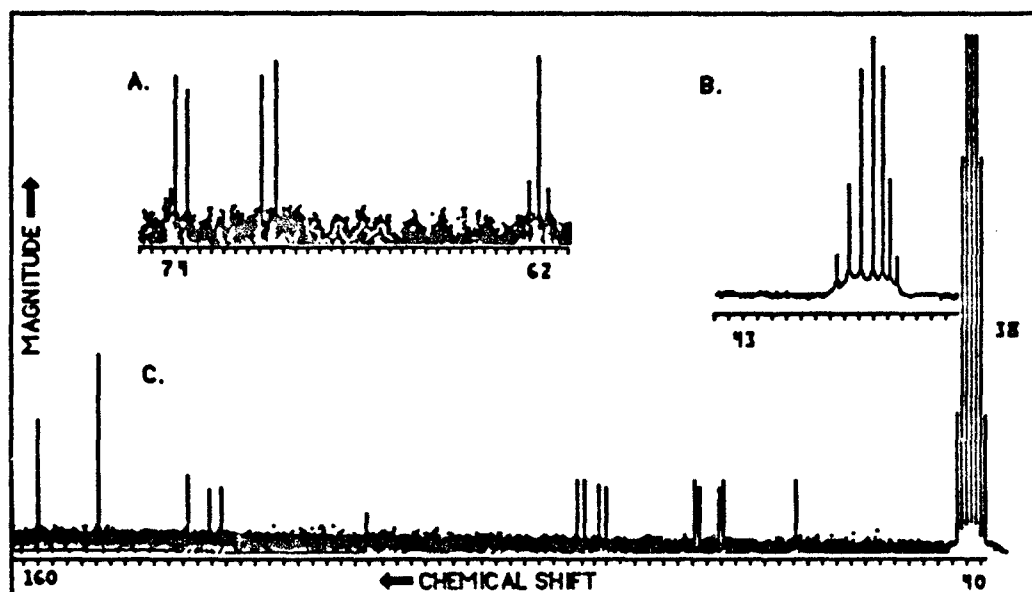


Figure 43.  $^{13}\text{C}$  proton off-resonance decoupled spectrum of xanthosine dihydrate.

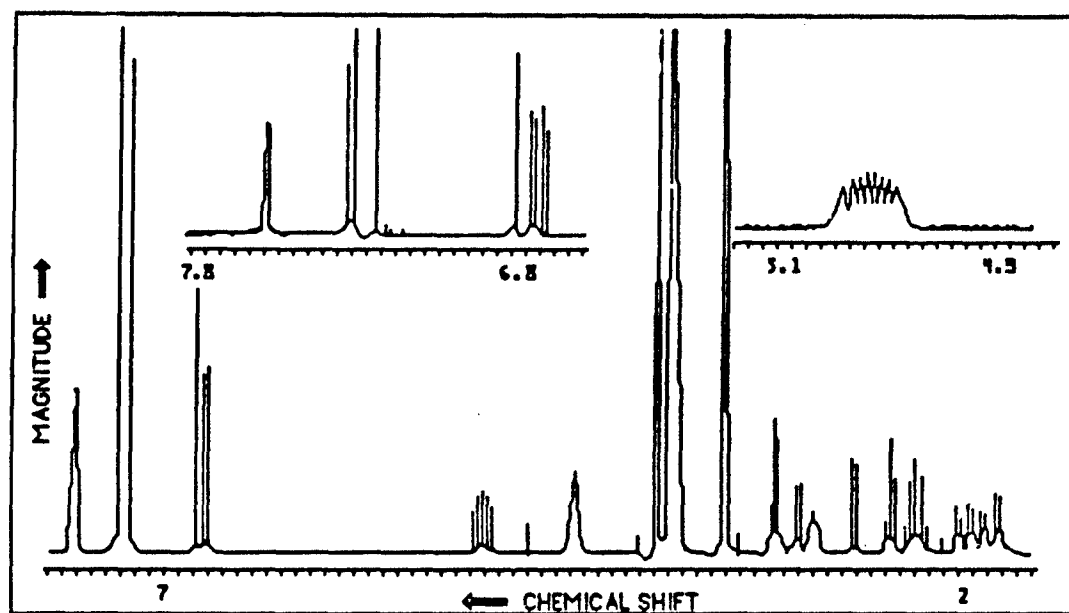


Figure 44. Proton coupled 500 MHz spectra of reserpine. Upper left insert shows low field region, the resonances are the two ring structures. Upper insert right shows the 8 lines of the C18H multiplet.

Reserpine,  $C_{33}H_{39}N_2O_9$  is the last compound analyzed in this reporting period. Having a molecular weight of about 600, it borders on the threshold of having a complete  $^1H$  NMR spectra. Considerably more time was required to analyze this molecule in comparison to the others in this report. The most complicated region of spectra for molecules of this type, the methylene region for 2.7 to 1.0 PPM was completely resolved. All resonances could be analyzed and coupling constants measured, thus attesting to the high resolving power of the VXR-500 instrument.

The proton decoupled  $C^{13}$  spectra is shown in Figure 42, where all 10 carbon atoms resolve separately, a  $C^{13}$  proton off-resonance decoupled spectrum is shown in Figure 43, identifying quarternary carbons at 158, 150, 140, and 116 PPM. These resonances being singlets. The mid-field carbons resolve as doublets, thus identifying themselves as CH carbons with the exception of C5 at 61 PPM which resolves as a triplet, more clearly seen in the upper insert.



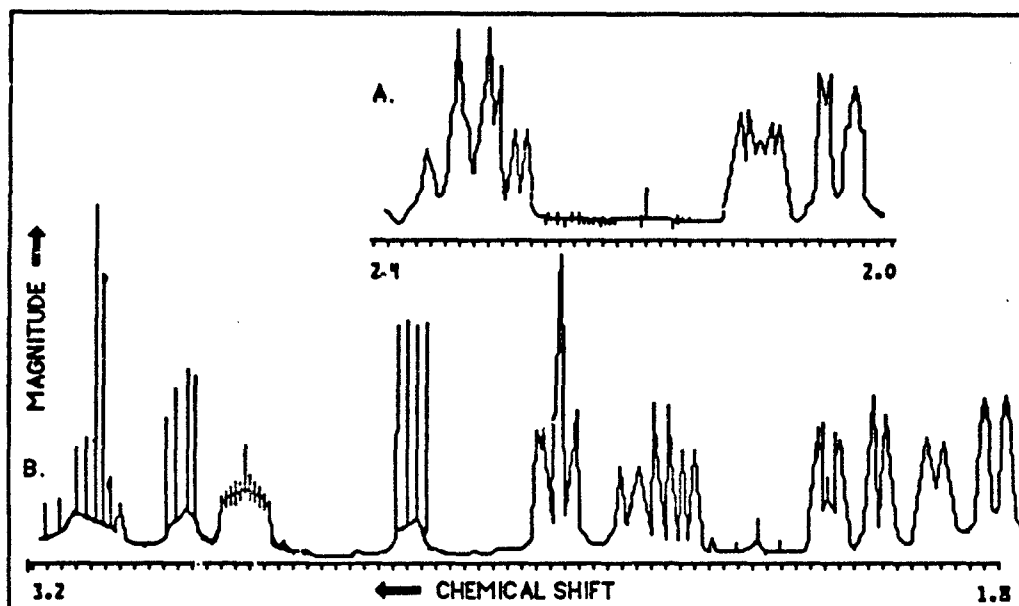


Figure 45. Proton coupled 500 MHz spectra of reserpine. 1.8 to 3.2 PPM region of proton spectra with 5.02 (C18H) decoupled. Apparent changes in the spectrum are seen at 2.30 (C19AH) and 1.97 (C19BH) thus identifying each of the protons.

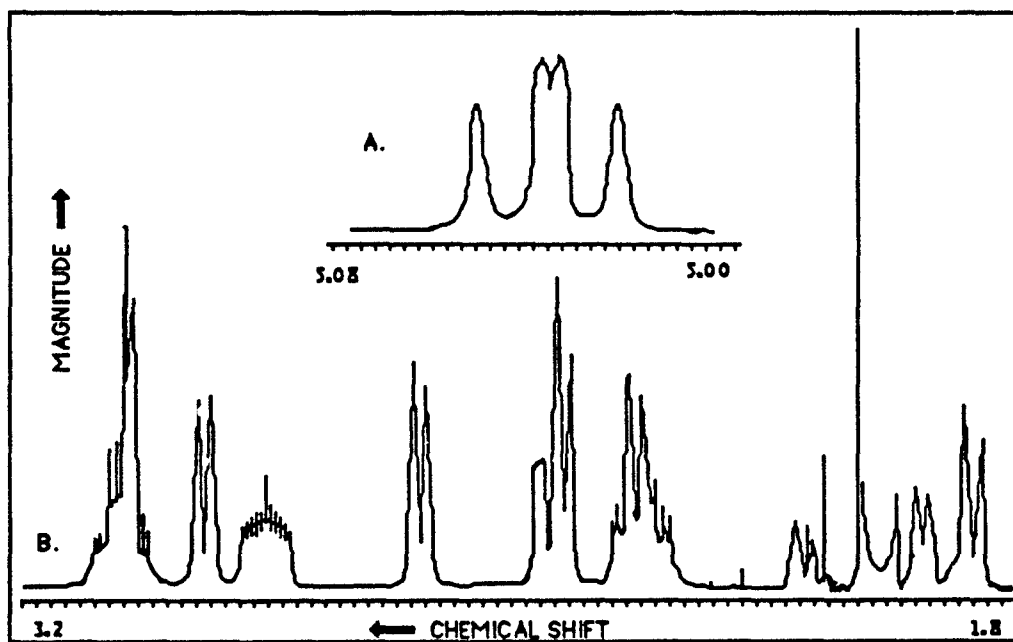


Figure 46. Proton decoupled 500 MHz spectra of reserpine. 1.8 to 3.2 region of spectrum with 1.97 (C19BH) decoupled. Note change in 2.04 resonance (C14H). Apparent partial saturation of 2.04 accounts for small change in 2.68 (C16H). Upper insert shows 5.02 (C18H) with coupling constants of 11.8 and 9.6 Hz. The 5.0 Hz coupling constant is due to C19BH.

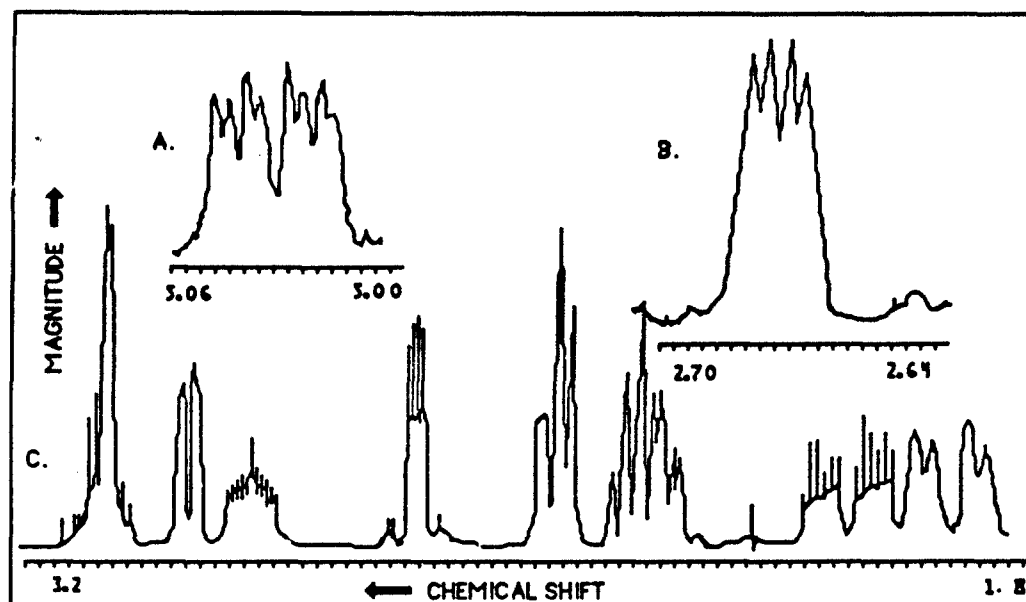


Figure 47. Proton decoupled 500 MHz spectra of reserpine. 1.8 to 3.32 spectral region with 3.89 (C17H) decoupled. Apparent changes in the spectrum are seen at 2.68 (C16H) and 5.03 PPM shown in upper left insert. Upper right insert shows 2.68 resonance having lost the large 11.2 Hz splitting due to C17H at 3.89.

One peak however, on first analysis, did escape detection. It turned out that one proton resonance was under the large methyl resonance at 3.90 PPM; undetected at first due to small artifacts normally found near the base of a large peak. This peak turned out to be C17H, coupled to both 5.02 and 2.68. When 3.90 was saturated, 5.02 and 2.68 both changed. This is shown in Figure 47. This was further confirmed by integration of the methyl peaks.

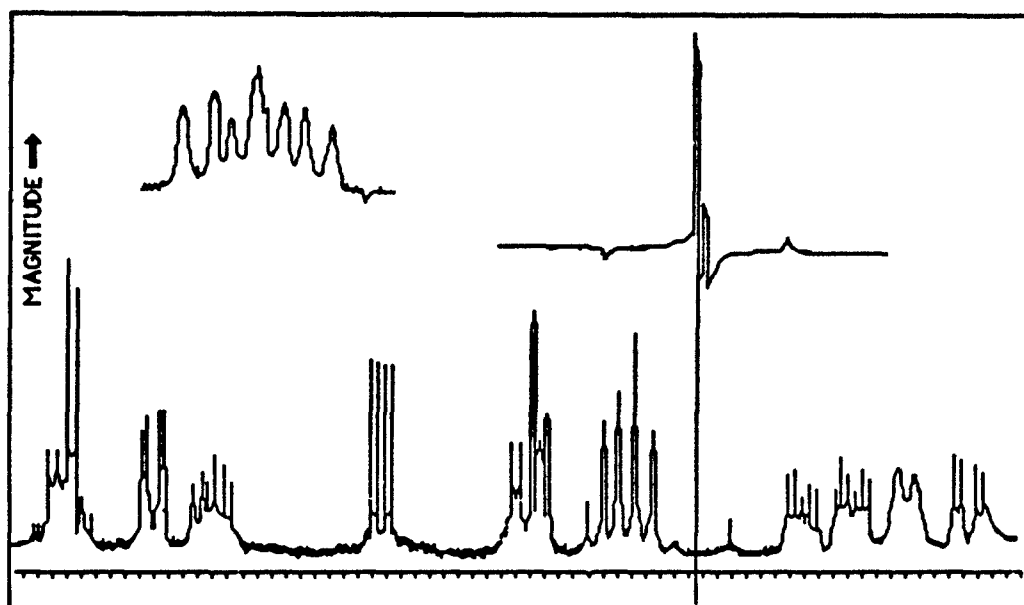


Figure 48. Proton decoupled 500 MHz spectra of reserpine. Decoupling C11H at 4.44 PPM, reveals changes in the spectrum at 2.29 (C12AH) and C12B (1.78). A small coupling is removed from C10B at 2.93 PPM. The upper left insert reveals no change in C18H at 5.02.

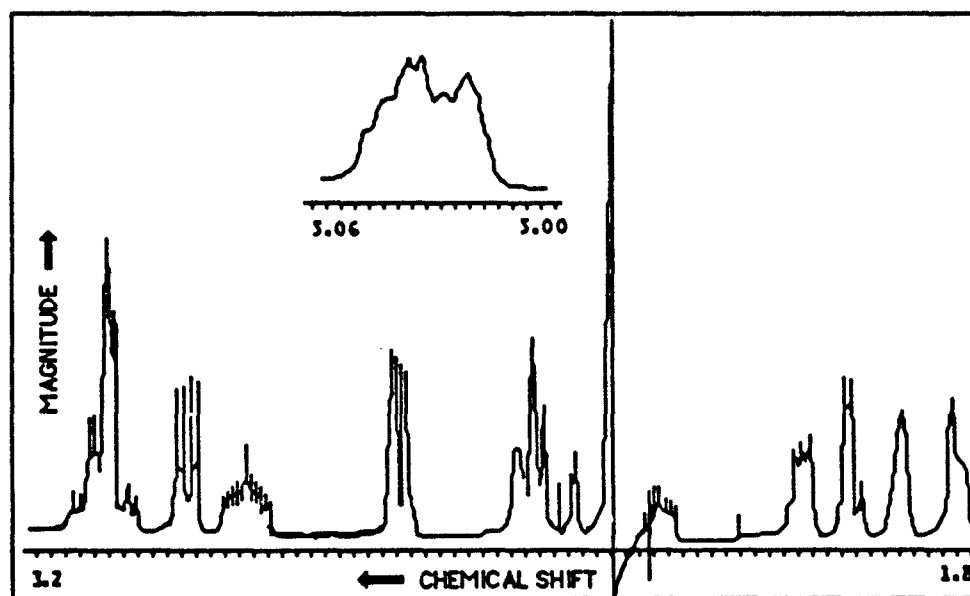


Figure 49. Proton decoupled 500 MHz spectra of reserpine. 1.8 to 3.32 spectral region with 2.34 decoupling.

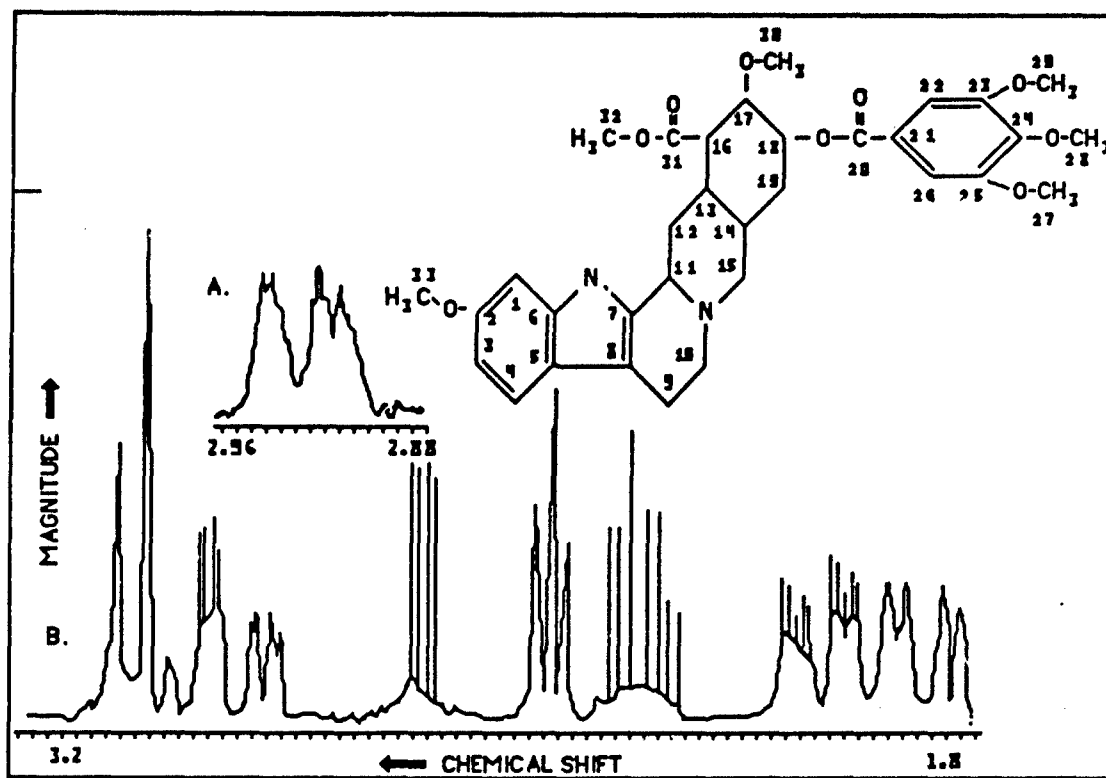


Figure 50. Proton decoupled 500 MHz spectra of reserpine. Decoupling at 3.14 (C10AH) produces change at 2.93 (C10BH).

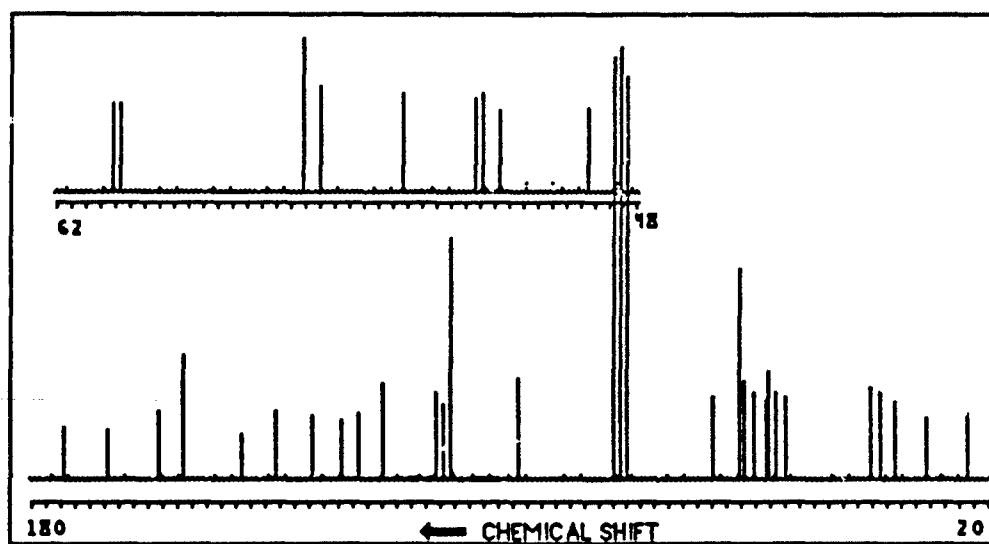


Figure 51. 300 MHz  $^{13}\text{C}$  NMR spectrum of reserpine.

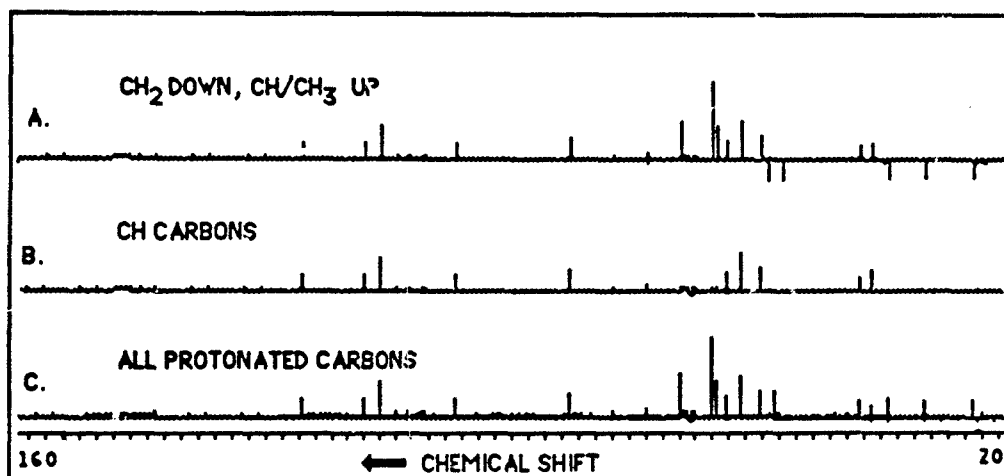


Figure 52. Distortionless, enhancement polarization transfer plot of reserpine.

The proton spectral assignments made for reserpine are as follows:

#### ASSIGNMENTS

NH	C4H	C22,26H	C2H	C3H	C18H	C11H	C17H	C27,28,29H3	C33H3	C32H3
7.57	7.31	7.30	6.82	6.75	5.02	4.44	3.90	3.89	3.82	3.80
C30H3	C10AH	C9AH	C15AH	C10BH	C16H	C9BH	C15BH	C19AH	C12AH	
3.48	3.17	3.5	3.03	2.93	2.68	2.46	2.44	2.33	2.29	
C13H	C19BH	C14H	C12BH							
2.04	1.97	1.88	1.78							

#### <sup>1</sup>H-<sup>1</sup>H COUPLING CONSTANTS

J3,4	J1,3	J9A,9B	J9A,10A	J9A,10B	J9B,10B	J10A,10B	J11,10B	
8.5	2.1	3.9	11.2	6.5	15.8	10.8	2.2	
J11,12A	J11,12B	J12A,12B	J12A,13	J12B,13	J13,16	J16,17	J17,18	
6.0	1.0	14.4	13.5	3.8	4.7	11.0	9.6	
J18,19A	J18,19B	J19A,19B	J19A,14	J19B,14	J14,15A	J15A,15B	J22,26	
11.8	5.0	13.0	12.4	4.0	3.3	11.0	1.1	

•EXAMINATION OF *G. TOXICUS* STRAINS We have been examined several aspects of toxin production. Specifically (1) acclimating 10 clones of *G. toxicus*, (2) treatment of these same ten clones with antibiotics to produce axenic cultures, (3) conducting test to develop a defined medium, (4) examination of the phosphate-ammonia-nitrate utilization by dinoflagellates, and (5) testing of sea water samples from Florida Institute of Technology.

The work with the acclimation of *G. toxicus* cultures is summarized as follows:

TABLE 7  
ACCLIMATION OF *G. TOXICUS* CLONES IN CULTURE

	175	350	135	177	163	158	157	199	169	619
Culture	8/5	8/5	8/5	8/24	8/8	8/5	8/5	8/8	8/5	8/5
interval	9/2	9/2	9/4	9/21	9/14	9/9	9/9	9/4	9/9	9/4
Div./Day	.20	.10	.12	.15	.17	.07	.08	.12	.11	.11
Yield mg**	(1)	(1)	127	141	164	152	166	118	182	113
Culture	9/2	9/2	9/4	9/21	9/14	9/9	9/9	9/4	9/9	9/4
interval	9/30	10/9	10/9	11/5	10/7	10/9	10/19	10/7	10/22	
Div./Day	.24	.11	.11	.11	.12	.12	.08	.12	.10	.08
Yield mg*	93	371*	457*	141	237*	559*	281*	272*	243*	113
Culture	9/18	9/28	9/25	10/22	9/25	9/28	9/28	9/28	9/25	10/8
interval	10/19	11/12	11/5	12/3	11/5	11/5	11/5	11/5	11/5	
	11/25									
Div./Day	.27	.10	.17	.09	.12	.11	.10	.12	.10	.07
Yield mg*	269	?	247	?	189	179	172	110	162	?
Culture	10/29	10/22	10/19	10/22	10/22	10/22	10/22	12/26	10/22	
interval	11/16									
	11/23	?	12/3	12/3	12/3	?	12/3	12/3	12/3	?
Div./Day	.27	.13	.17	?	.18	.12	.14	.16	.12	
Yield mg*	269	?	247	?	189	179	172	110	162	?

Explanation of remarks: (1) =used as inoculum, \* =lypholized with sea water instead of distilled. Bold Division rate indicated organism put into 15L culture.

Table 7 above is a record of 1.5L and 15L cultures in the acclimated cell toxicity procedures. It provides a record of: the size of the culture, culture interval, reproduction rate (Div/Day) and final

cell yield (mg dry weight). In September and October some samples were lyophilized with seawater to try to limit leaching of cell contents during processing (values marked with \*). This resulted in substantial increases in dry weights which are misleading. We returned to freeze drying with distilled water in later September and October cultures. It was found that if processing was rapid enough leaching could be minimized.

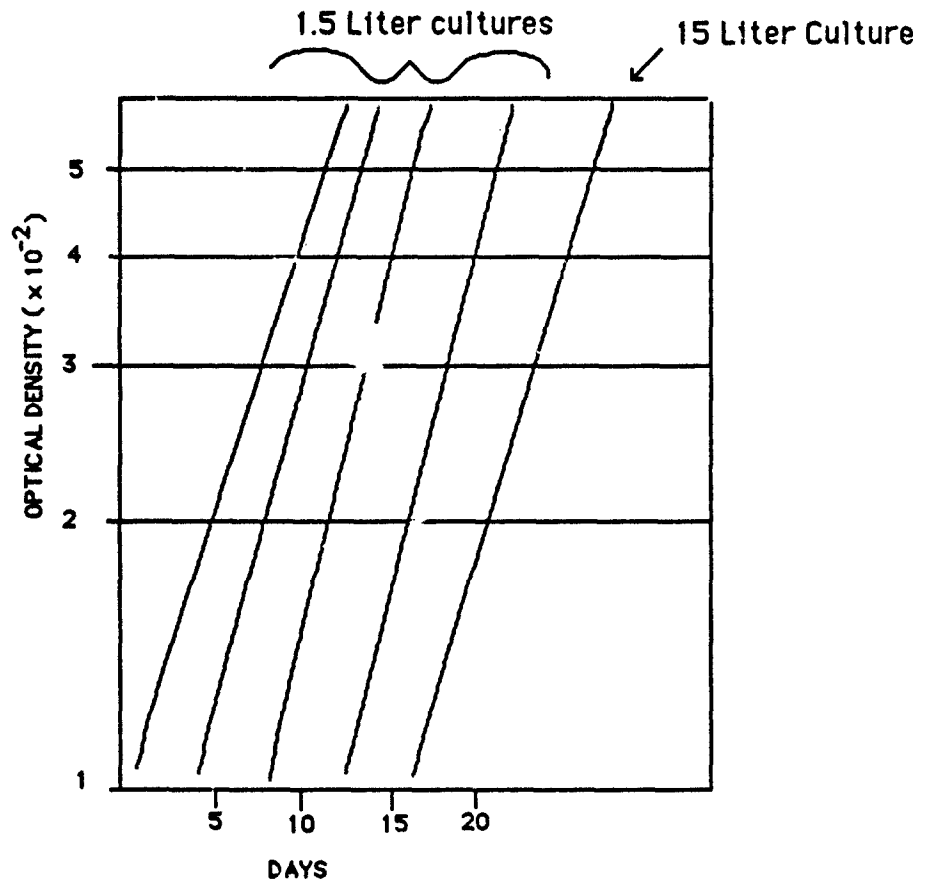


Figure 53. Acclimation of clone 175. Clones were acclimated in 1.5 liter cultures until growth rates became approximately constant and then were moved into 15 liter cultures.

The above data indicate that there has been a general increase in division rate and cell yield thus indicating that acclimation is occurring. In judging this successive growth plots (log phase slopes) of each clone are plotted. When two to three successive slopes align (Figure 53), then the culture is deemed to be acclimated. In this

respect, clones 175 and 135 have been deemed to be acclimated and were, therefore, moved to 15 liter cultures.

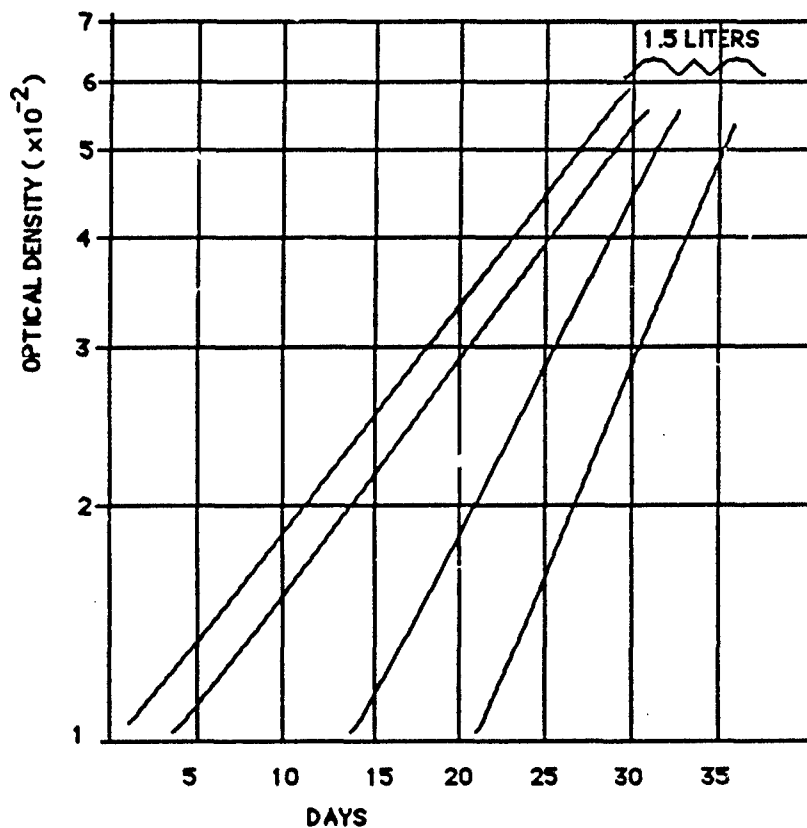


Figure 54. Acclimation of clone 135. Clones were acclimated in 1.5 liter cultures until growth rates became approximately constant. Clone 135 was moved into 15 liter culture on 30 November 1985.

Treatment with antibiotics progress. Clone 175 is now in axenic (bacteria free) culture after treatment with penicillin and streptomycin.

Defined medium testing. A new defined medium has been developed which fosters excellent growth and morphology of *G. toxicus*. The medium is called JW-2. At present it is undergoing testing to determine its effect on the toxicity of *G. toxicus* and what other clones will grow in it.



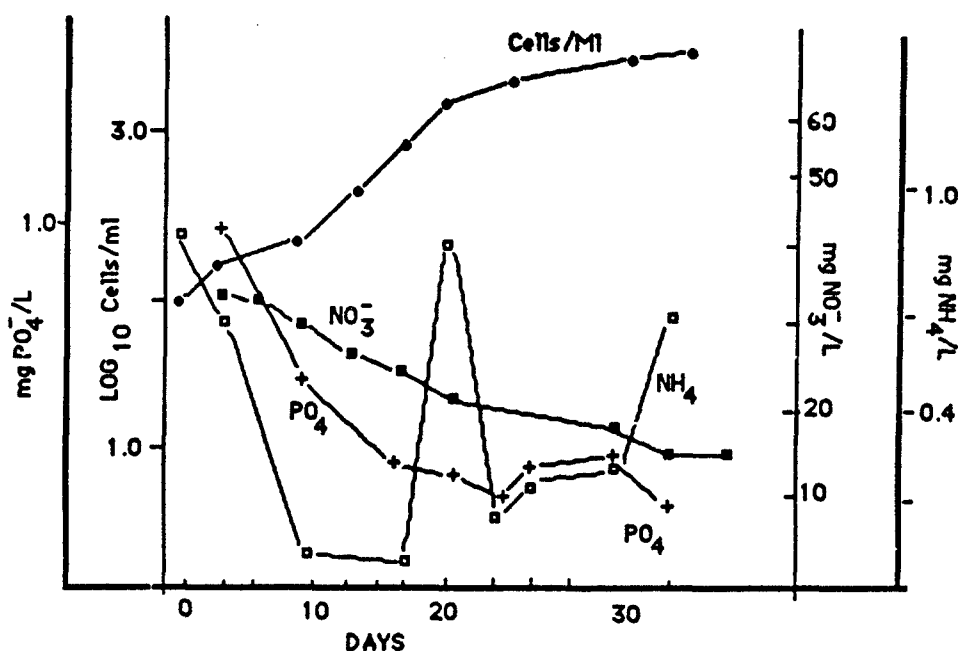


Figure 55. Characteristics of growth of *Gambierdiscus toxicus* (clone 175). Four parameters, cells per ml, nitrate, phosphate and ammonia are plotted as a function of time.

Phosphate-ammonia-nitrate utilization. Studies completed on *G. toxicus*, clone 175, indicate that both phosphate and ammonia are readily taken up by the rapidly dividing cells (see Figure 55). The pulse of ammonia at 19 days of culture is apparently real, having shown up in four separate cultures to date. The cultures begin to foam at this point, indicating a general increase in the production of extracellular metabolites. Cultures are currently being grown for extraction of the medium to determine if toxin is lost from cells during this "pulse" period. Future work will involve manipulating the N/P atomic ratios in attempts to improve toxin production.

Florida sea water tests. Growth plots of four toxigenic species (*Prorocentrum lima*, *Coolia monotis*, *G. toxicus* and *Ostreopsis* sp) indicate that the sea water samples we received from Florida Institute of Technology is as good if not better than the water we receive from Carolina Biological Supply Company.

ACQUISITION AND MAINTENANCE OF CULTURE COLLECTION This year we have been especially successful in adding to our culture collection. Our activities in this respect are best summarized in Tables 8A, B, and C on the next three pages.

TABLE 8., PART A  
THE SOUTHERN ILLINOIS UNIVERSITY (SIU) CULTURE COLLECTION  
OF DINOFLAGELLATES FROM THE CARIBBEAN (AND ELSEWHERE).

Species	SIU Strain or Clone Number	Origin
<i>Amphidinium asymmetricum</i>	831	British Columbia, Canada *
<i>Amphidinium carterae</i>	839	South Sound, Virgin Gorda *
<i>Amphidinium carterae</i>	810	R. R. L. Guillard *
<i>Amphidinium elegans</i>	547	Virgin Gorda *
<i>Amphidinium sp.</i>	MB005	Florida Keys *
<i>Amphidinium klebsii</i>	83, 101, 104, 126, 145	Knight Key, Florida **
<i>Amphidinium sp.</i>	54, 59, 60, 61	Knight Key, Florida **
<i>Cochlodinium polykrikoides</i>	208, 489	South Sound, Virgin Gorda *
<i>Coolia monotis</i>	263	Hurricane Hole, St. John *
<i>Coolia monotis</i>	390	South Sound, Virgin Gorda *
<i>Coolia monotis</i>	838	Virgin Gorda *
<i>Coolia monotis</i>	602	Grosse Caye, Haiti *
<i>Coolia monotis</i>	106, 140, 147, 150, 180, 188	Knight Key, Florida **
<i>Ensiculifera carinata</i>	415	South Sound, Virgin Gorda *
<i>Gambierdiscus toxicus</i>	350, 467, 850	South Sound, Virgin Gorda *
<i>Gambierdiscus toxicus</i>	842	Virgin Gorda *
<i>Gambierdiscus toxicus</i>	900, 901, 902	Biras Creek, Virgin Gorda *
<i>Gambierdiscus toxicus</i>	851, 852	Greater Lameshur Bay, St. John *
<i>Gambierdiscus toxicus</i>	853	Little Lameshur Bay, St. John *
<i>Gambierdiscus toxicus</i>	619	St. Thomas Lagoon, St. Thomas *
<i>Gambierdiscus toxicus</i>	117, 130, 131, 136, 195, 196, 198, 199	Knight Key, Florida **
<i>Gambierdiscus toxicus</i> <sup>1</sup>	135, 190, 192	Bermuda **
<i>Gambierdiscus toxicus</i>	157, 158, 160, 164, 165, 191	Bahamian Drift Algae **
<i>Gambierdiscus toxicus</i>	196	Northeast Rock, Bahama Drift **
<i>Gambierdiscus toxicus</i>	172, 173	Great Isaacs Light, Bahamas **
<i>Gambierdiscus toxicus</i>	159, 162, 163, 166, 168, 170, 171, 193	Gingerbreads, Bahamas **
<i>Gambierdiscus toxicus</i> <sup>2</sup>	175 (MQ-1), 176 (MQ-2)	Martinique **
<i>Gambierdiscus toxicus</i> <sup>3</sup>	177 (T-39)	"Hawaiian Strain" **

**TABLE 8., PART B**  
**THE SOUTHERN ILLINOIS UNIVERSITY (SIU) CULTURE COLLECTION**  
**OF DINOFLAGELLATES FROM THE CARIBBEAN (AND ELSEWHERE).**

Species	SIU Strain or Clone Number	Origin
<i>Gonyaulax grindleyi</i>	403	Hurricane Hole, St. John *
<i>Gonyaulax grindleyi</i>	781	Drake's Channel, St. Thomas *
<i>Gymnodinium sanguineum</i>	373, 374, 497	South Sound, Virgin Gorda *
<i>Gymnodinium</i> sp.	74, 76, 77, 78, 81, 82	Knight Key, Florida **
<i>Gyrodinium fissum</i>	376, 379, 474	South Sound, Virgin Gorda *
<i>Ostreopsis lenticularis</i>	702	St. Thomas Lagoon, St Thomas *
<i>Ostreopsis lenticularis</i>	841	Virgin Gorda *
<i>Ostreopsis lenticularis</i>	870, 871, 872, 873, 875	South Sound, Virgin Gorda *
<i>Ostreopsis lenticularis</i>	874	Biras Creek, Virgin Gorda *
<i>Ostreopsis lenticularis</i>	875a, 876b	Little Lameshur Bay, St. John *
<i>Ostreopsis heptagonia</i>	200, 201, 202, 203, 204, 206, 207, 208, 209, 210, 211, 212, 213, 214, 219	Knight Key, Florida **
<i>Prorocentrum concavum</i>	364	Salt Island *
<i>Prorocentrum concavum</i>	843	Virgin Gorda *
<i>Prorocentrum concavum</i>	881	Anegada *
<i>Prorocentrum concavum</i>	882a, 882b	Little Lameshur Bay, St. John *
<i>Prorocentrum concavum</i>	883	Greater Lameshur Bay, St. John *
<i>Prorocentrum lima</i>	700	South Sound, Virgin Gorda *
<i>Prorocentrum lima</i>	844	Virgin Gorda *
<i>Prorocentrum lima</i>	885	Little Lameshur Bay, St. John *
<i>Prorocentrum lima</i>	836	Unknown (cold water form) *
<i>Prorocentrum lima</i>	62, 105, 142, 178, 185, 186, 187	Knight Key, Florida **
<i>Prorocentrum micans</i>	825	British Columbia, Canada *
<i>Prorocentrum</i> sp.	MB130, MB134, MB157	Florida Keys *
<i>Prorocentrum</i> sp. nov.	MB136	Florida Keys *

TABLE 8., PART C  
THE SOUTHERN ILLINOIS UNIVERSITY (SIU) CULTURE COLLECTION  
OF DINOFLAGELLATES FROM THE CARIBBEAN (AND ELSEWHERE).

Species	SIU Strain or Clone Number	Origin
<i>Prorocentrum mexicanum</i>	262, 273, 276	Salt Island *
<i>Prorocentrum mexicanum</i>	722	Hurricane Hole, St. John *
<i>Prorocentrum mexicanum</i>	840	Virgin Gorda *
<i>Prorocentrum mexicanum</i>	880	Biras Creek, Virgin Gorda *
<i>Prorocentrum mexicanum</i>	884	Greater Lameshur Bay, St. John *
<i>Prorocentrum mexicanum</i>	69, 84, 86, 181, 182, 183, 184	Knight Key, Florida **
<i>Scrippsiella subsalsa</i>	86, 404, 724,	Hurricane Hole, St. John *
<i>Scrippsiella subsalsa</i>	344	Biras Creek, Virgin Gorda *
<i>Scrippsiella trochoidea</i>	557, 582, 587	South Sound, Virgin Gorda *
<i>Symbiodinium microadriaticum</i> <sup>4</sup>	151, 152	Florida Keys **

<sup>1</sup> collected by Dr. L. E. Brand

<sup>2</sup> provided by Dr. J. Babinchak

<sup>3</sup> collected and provided by Dr. N. Withers

<sup>4</sup> collected by Mr. J. Thomas and Dr. J. Bomber

\* Original SIU Collection

\*\* Acquired from FIT Collection

DELIVERY OF TOXINS During the reporting period we delivered two shipments of crude toxin labeled as follows:

LOT #	KIND	CONTRACT ITEM	AMOUNT
Lot GT350-1A/87H	Crude	Item 0001 AL	13.8 mg
Lot GT350-1A/87H	Crude	Item 0001 AM	10.38 mg

The above two deliveries were optional crude requested by the Commander. The adjusted schedule for delivery of purified toxins begins in February 1988.

## SUMMARY OF RESULTS

- (1) Acquired personnel and equipment and setup the latter to accomplish the proposed tasks.
- (2) Thus far have grown approximately 1,246 liters of *G. toxicus* which has yielded 464.4 g wet weight of material. When dried down this has yielded 75.97 g of dry weight.
- (3) Conducted experiments on solvents, interacting factors and stability of the toxins from *G. toxicus*.
- (5) Conducted experiments on the separation and extraction of the toxins from *G. toxicus*.
- (6) Conducted over 220 ileum assays.
- (7) Conducted over 50 mouse bioassays.
- (8) Constructed and put into use a water bath device for the acclimation of dinoflagellate cultures.
- (9) Conducted daily experimentation on ten different clones of *G. toxicus* to achieve acclimation preliminary to comparing relative toxicity between clones.
- (10) Utilized 103 hours of NMR time in the examination of 30 model compounds.
- (11) Establishment of one axenic clone of *G. toxicus*.
- (12) Conducted experiments on the relationship between phosphate-ammonia-nitrate utilization relative to toxin production.
- (13) Increased the culture collection by 50%.
- (14) Tested alternate sources of sea water for use.
- (15) Developed an artificial sea water for *G. toxicus*.
- (16) Delivered 24.18 mg of crude toxin extract to USAMRIID.

## DISCUSSION

We have acquired the personnel and set up the equipment to accomplish the stated objectives. To the limit that our available facilities have allowed we have grown the dinoflagellate *G. toxicus* and productivity has been as predicted. We have at the same time incorporated studies on growth, purification, extraction and storage which have produced publishable data. In terms of the cost benefit ratio, our studies on extraction and purification are paying off in terms of increased toxin production per gram of organism. The studies on the different clones of *G. toxicus* have indeed established that there are significant differences between strains in terms of toxicity. The development of axenic cultures in a defined medium is especially important in toxin production. In the area of toxin assay, we have been able to improve the number of tests completed per ileum to three. This has allowed us to reduce the number of mouse assays and because of the sensitivity of the ileum, saved large amounts of toxin. Finally, our work in the Caribbean this last summer has resulted in important data and additions of new toxic clones for the culture collection.

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**GLOSSARY**

ACN-insoluble	Acetonitrile insoluble fraction
ACN-soluble	Acetonitrile soluble fraction
DEPT	Distortionless Enhancement Polarization Transfer Plot
DMSO	Dimethyl sulphoxide
ESAF	Ether soluble acetone filtrate
ESAP	Ether soluble acetone precipitate
HPLC	High Pressure Liquid Chromatography
NMR	Nuclear Magnetic Resonance
PPM	Parts per million
PSS	Physiological Saline Solution
TEA	Tetraethylammonium
WSAF	Water soluble acetone filtrate
WSAP	Water soluble acetone precipitate

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